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Search for New Inhibitors of Human Aromatase Enzyme (Cyp450) from Bioactive Compounds of *Citrus* species

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ABSTRACT

Oestrogen synthesis pathway is one of the bottom line steps for breast cancer advancement; involving, aromatase enzyme (Cyp450), which transform androgens to oestrogens. Thus endocrine-based therapies comprising of human aromatase blockage is the most necessary way in order to decrease the oestrogen levels and thereafter prohibiting the chances of breast cancer commencement. In recent years, limelight on drug discovery from green sources has been growing for their less toxicity and cost effectiveness. Our present course of study aims at searching of new antagonist/s from a common dietary source *"Citrus species"*. Molecular docking along with *In-silico* evaluation their pharmacokinetics (ADME) properties and toxicity were employed to fulfill the aim. Result shows that, all the five Citrus compounds have reasonable affinity towards cytochrome p450. However, Hesperidin shows highest affinity towards its target receptor protein i.e. -9.7 kcal/ mol, followed by Chalcone that shows the lowest affinity towards its target protein i.e. -7.4 kcal/ mol. Hence, bioactive components of *Citrus* species can be green alternatives for breast cancer therapy.

Keywords: ADME, Aromatase, Bioactive compounds, Breast Cancer, *Citrus* species, Cyp450.

INTRODUCTION

Aromatase (gene CYP19) belongs to the cytochrome P450 enzyme family that synthesizes oestrogens from androgens. This gene expression was regulated differently in various tissues including breast, skin, adipose tissue and brain and so on[1-8]. In case of breast cancer, oestrogen levels have been found to be elevated due to malfunctioning of aromatase enzyme. Hence, one of the efficient contemporary therapeutic approaches to dealing with oestrogen-dependent breast cancer could be aromatase suppression[9-11], using inhibitors. Aromatase inhibitors are of three different generations based on their evolutionary time scale[12,13]. In comparison to existing breast cancer therapies; this aromatase inhibiting mechanism via plant sources exhibit better efficacy with fewer side effects[14,15]. With respect to herbal alternative meditative approach,[16-18] *Citrus* plant is one of the well-recognized, worldwide cultivated, due to its enormous healing and health promoting qualities[19, 20]. Among all the plentiful active secondary metabolites of *Citrus* species, here in our study, we have selected five bioactive compounds viz. Naringenin, Hesperidin, Apigenin, Luteolin and Chalcone as our ligands for the *In-silico* prediction of inhibitors against human aromatase enzyme. Naringenin, shows its

beneficial activities mostly by its antioxidant actions[21-24]. Additionally, it has been shown to adhere to estrogen receptors[25,26], showing that it has effects in both directions[27-31]. It is most often used for haemorrhoids; besides this, it possesses antioxidant and anti-inflammatory effects[32-34]. Apigenin belongs to the class of flavonoids that generally increases[35] antioxidant enzymes like SOD, CAT and glutathione peroxidase[36-38] and decreases the generation of reactive oxygen species[39]. Luteolin belongs to flavone[40]; it possesses differential human health beneficial properties starting from anti-antioxidant properties to anti-inflammatory, anti-diabetic as well as anti-carcinogenic potential[41]. Chalcone belongs to the group of aromatic ketone collectively known as chalcones or chalconoids, largely found in *Citrus* species. Numerous biological functions are manifested by chalcones and their derivatives[42,43]. They have been identified as the potential anti-infective candidates that inhibit various microbial communities[44]. Essential oils, isolated from *Citrus* plants also used for aromatherapy that make it to be utilised as an alternative medicine[45]. Based on our above mentioned literature review, our purpose of work is to investigate *In-silico* ligand oriented protein selection along with molecular comparison on the basis of computational assessment of different pharmacokinetic, toxicity profile of five well known bioactive components from *Citrus* species. The overall schematic workflow is given in Fig. 1 as a graphical abstract.

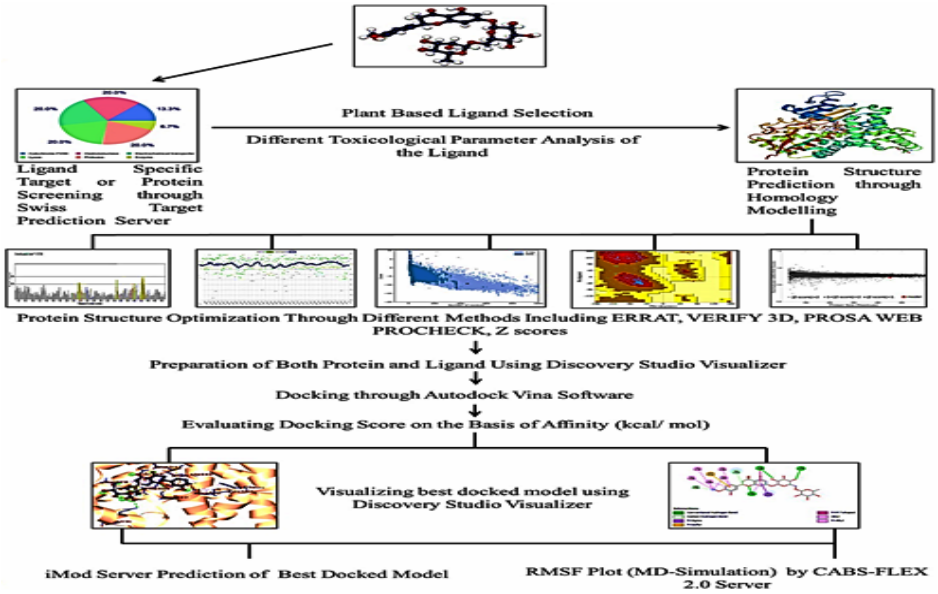

Fig. 1. The Schematic Workflow

MATERIALS AND METHODS

Selection and Preparation of Ligands

Chemical compounds or rather more accurately termed as ligands were selected on the basis of literature study from online database server "IMPPAT' (https://cb.imsc.res.in/imppat/). After the review work, total five bioactive compounds from *Citrus* species were selected to be used as ligands: Naringenin, Hesperidin, Apigenin, Luteolin and Chalcone. Fig. 2 the 2D Structure Data Format (SDF) of all the ligand structures was downloaded from online chemical database: PubChem (www.pubchem.ncbi.nlm.nih.gov) and eventually was converted to their respective 3D PDB format[46]. A PDBQT format file was created by adding hydrogenating atoms and the desired torsion to a PDB format file.

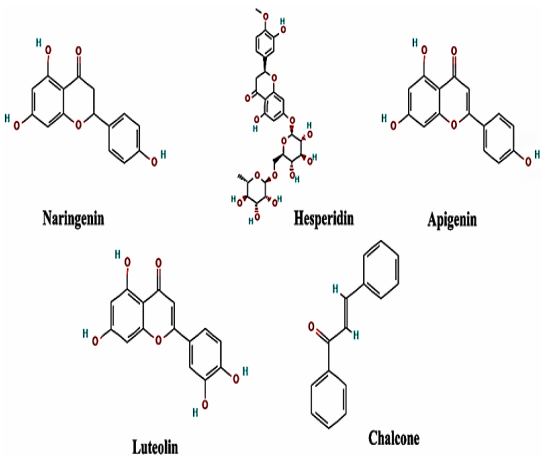

Fig. 2. 2D Structures of Chemical Compounds from *Citrus* Species

Selection of Receptor or Proteins

For the purpose of ligand-based protein selection, SMILEs of all the previously mentioned compounds were put into Swiss target prediction server (www.swisstargetprediction.ch/)[47]. For each and every compound, a pie diagram (Fig. 3A-3B) showing their affinity towards various classes of proteins was generated.

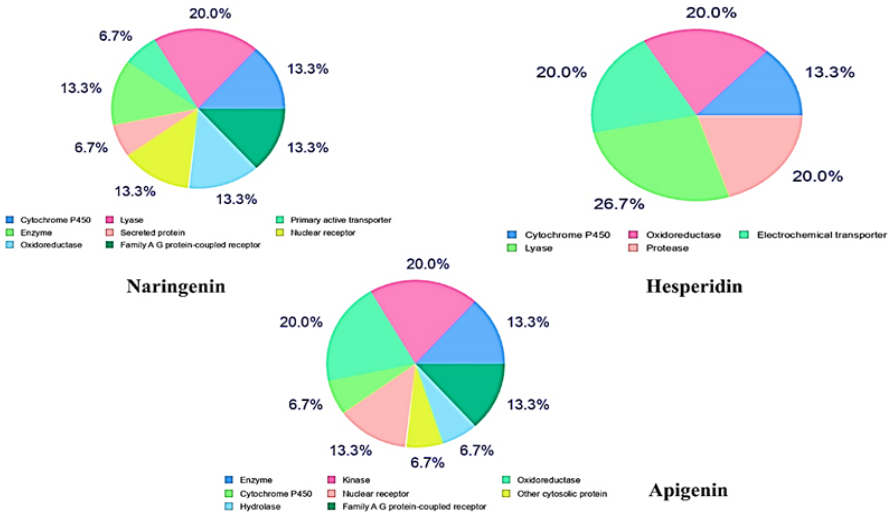

Fig. 3A. Pie Charts Showing Result of Compound Mediated Swiss target Prediction

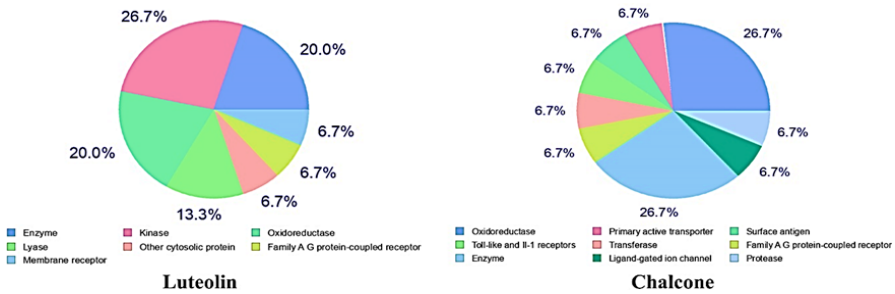

Figure 3B: Pie Charts Showing Result of Compound Mediated Swiss target Prediction

Protein Preparation

Based on the target prediction data form online Swiss target prediction server, the crystal structure of human aromatase cytochrome p450 i.e. 3EQM Fig. 4 has been selected for the study. The 3D structure has been retrieved from Protein Databank (http://www.rcsb.org/). Then, using BIOVIA Discovery Studio 2020 software, the already-attached compounds and water molecules were eliminated in order to stabilize the receptor structures. (https://discover.3ds.com/discovery-studio-visualizer-download/)[48].

Validation of Protein Structure

Each new retrieved protein PDB structure was then undergone through a series of quality analyses including ERRAT, Verify 3D, Procheck using SAVES 6.0 (https://saves.mbi.ucla.edu/), QMEAN-Z-score using SWISS-MODEL server (https://swissmodel.expasy.org/qmean/)[49-52] and ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php/)[53].

Fig. 4. 3D Structure of Human Aromatase Cytochrome p450 Protein (3EQM)

Toxicity Prediction of the Compounds

In case of drug designing and establishment of a suitable drug compound, it is a very necessary step to predict the toxicity level of the small compounds or rather ligands before investigating their endurance capacity when ingested into any animal model like mouse, rat as well as in human too. There are two online servers available for these purposes, they are: Adsorption, Distribution, Metabolism and Excretion all-together termed as ADME is a very profitable process to assess all those previously mentioned parameters of the ligands using the server of SwissADME website (https://www.swissadme.ch/)[54,55] and PreADMET server (https://preadmet.bmdrc.kr/)[56,57].

Bioactivity Score Prediction Using Molinspiration Chemoinformatics Tool

Molinspiration chemoinformatics online tool (https://www.molinspiration.com/) helps to identify different bioactivity scores of a compound only upon submitting its SMILE structure to the server. Six various bioactivity scores can be predicted by supporting molecular manipulation, fragmentation, processing and conversion[58].

Molecular Docking Interaction Using AutoDock Vina

AutoDock Vina software (http://vina.scripps.edu/)[59] for molecular docking and virtual screening that significantly improves efficient binding mode predictions, thereafter gives more accuracy in protein-ligand interaction. AutoDock Vina works by calculating the grid maps and clusters. Kollman charges and other changes were made to the purified form of the protein before moving on to the final docking phase and converting it into a properly readable PDBQT file format. The ligand is similarly converted into a PDBQT file. A grid box on the protein's active residues was created, with various grid sizes and centres, but a consistent grid spacing of 0.375. With AutoDock Vina software, binding energy affinity was predicted with an exhaustiveness of 8 set. Using BIOVIA Discovery Studio 2020, the docked structure's final depiction was carried out (https://discover.3ds.com/discovery-studio-visualizer-download/)[48] and PYMOL software (https://pymol.org/)[60].

Assessment of Active Amino Acid Residues or Structural Hotspots on the Receptor Protein

In order to predict active amino acid residues or in other words structural hotspots on the receptor protein, an online server known as CASTp 3.0 (http://sts.bioe.uic.edu/)[61] is used. A systematic quantitative characterisation of the surface topography of proteins is often provided by the Computer Atlas Surface Topography of Protein (CASTp).

iMod Server Prediction

The iMod server (http://imods.chaconlab.org/)[62] enables the investigation of such modes and generates workable transition paths between two homologous structures, even with huge macromolecules. The server prediction provides a number of analytics for users to interpret, including the iMod interaction status between docked protein-ligand structures, Deformability, B factor/mobility, Eigenvalue, Covariance Map, and Elastic Network.

CABS-flex 2.0 Server prediction

Process of MD-Simulation is carried out in CABS flex 2.0 webserver (http://biocomp.chem.uw.edu.pl/CABSflex2/) that is one of the well-known tools for fast simulation[63-65]. CABS-flex is better fitted for detecting non-obvious dynamic fluctuations, According to the result generated via the web server; the 'Fluctuation plot' tab provides an interactive 2D RMSF plot after global superposition.

RESULTS AND DISCUSSIONS

Validation of Protein Structure

The overall quality recognition of the 3D protein PDB structure i.e. 3EQM, as predicted by several previously mentioned online tools are represented through Fig. 5A-5B, 6 and Table 1. Approval of perfect protein structure is done through verifying the protein PDB model via a series of quality checking parameters. ERRAT showing results of "overall quality factor" for non-bonded atomic interactions, with higher scores indicating higher quality, according to ERRAT result, each of the protein exhibits their quality score more than 97.072%, which means each of protein is well modelled. Next coming to VERIFY 3D result which shows the result in the form of % of residues that have average 3d-1d score >=2; according to the server data, it shows 96.68% of residues bearing average 3d-1d score >=2. Next talking about ProSA-web result showed the overall z score of the protein; here the score is -9.24, which means the structure is under X-ray region. Next according to

the PROCHECK result, the Ramachandran plot of each protein model revealed that 86.6% of residues were present in the most favoured regions, followed by 11.6% in additional allowed, 1.5% in generously allowed and 0.2% in disallowed regions. Last but not the least, then quality comparison graph generated through QMEAN Server revealed range of quality in terms of Z score within a favourable region. All the results accumulated from these above mentioned parameters rectify that each of the protein has a good quality and suitable for studying further molecular interactions.

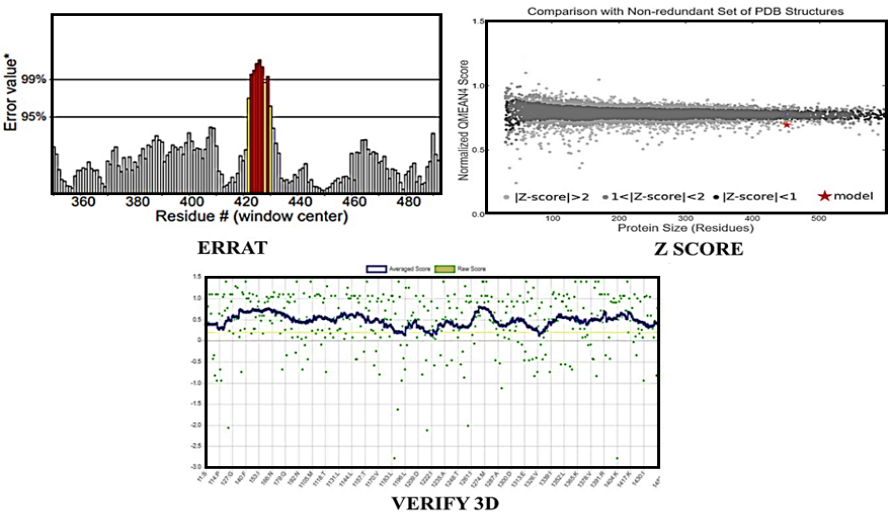

Fig. 5A. Quality Checking Parameters of Protein (3EQM) Structure

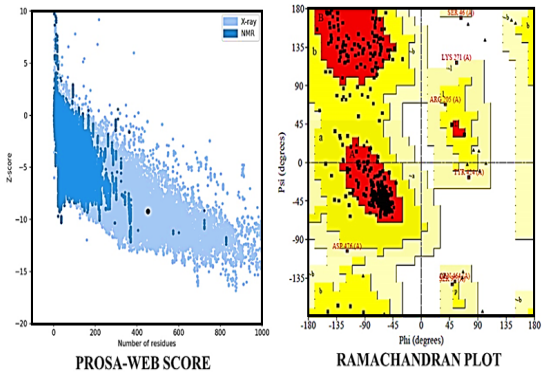

Fig. 5B. Quality Checking Parameters of Protein (3EQM) Structure

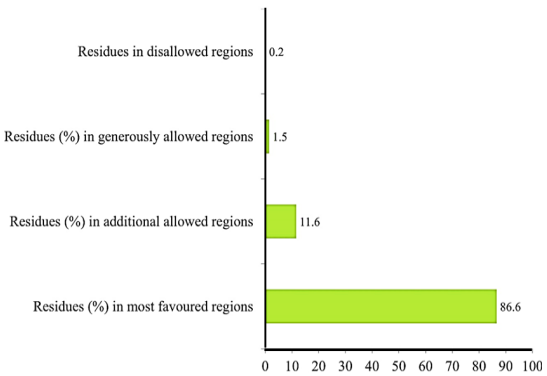

Fig. 6. Graph Showing the RAMACHANDRAN Plot Statistics

Table 1: Table Showing ERRAT, VERIFY 3D and PROSA WEB Scores of Protein 3EQM

Name of the protein	ERRAT Quality Score	VERIFY 3D% of Residues Having Average 3d-1d Score>=0.2	PROSA WEB Score
3EQM	97.072	96.68	-9.24

Results of Toxicity Prediction of the Compounds

The lack of pharmacokinetic research is one of the main obstacles to the commercialization of herbal remedies as medicines. Therefore, the study's objective was to assess the physicochemical, pharmacokinetic, and drug-likeness characteristics of *Citrus* species derived five bioactive components using computational approaches. Upon submission of compound structure in SMILEs format, SwissADME result is generated on the basis of Lipinski filter analysis. According to the result obtained via SwissADME server, it is observed that all the four compounds obey the Lipinski's rule of 5[66-67] and thereafter show satisfactory result. Next discussing about bioavailability score, all the four compounds show similar value of 0.55 i.e., all of them have 55% probability of being bioavailable[67].

In the process of developing new drugs, toxicity testing of small compounds is an essential phase. All four of the bioactive compounds found in *Citrus* species, except Hesperidin are mutagenic, according to the PreADMET server's toxicological prediction results, which take into account the chemical's hERG inhibition, mutagenicity, and carcinogenicity. Positive results indicate the compound has no carcinogenic activity, while negative results translate carcinogenic activity. According to the documentation, it can be observed that in case of rat, naringenin, apigenin and luteolin show no carcinogenic activity, whereas, hesperidin and chalcone show carcinogenic property. On the other hand, in case of all the five compounds show carcinogenic activity. Talking about the mutagenic characteristics, out of five compounds, except for hesperidin, all are mutagenic in nature. In case of hERG inhibition, except for hesperidin, which exhibit high risk, all other three compounds show medium probabilities of blogging hERG gene that often associated with sudden heart attacks in humans. The results of overall toxicity analysis data was represented via Table 2, 3 and 4.

Table 2: Table Showing Results of Drug Likeliness

Name of the compound	Lipinski's rule		
	Satisfactory	No. of violations	Bioavailability score
Naringenin	Yes	0	0.55
Hesperidin	No	3	0.17
Apigenin	Yes	0	0.55
Luteolin	Yes	0	0.55
Chalcone	Yes	0	0.55

Table 3: Table Showing Results of Mutagenicity and Carcinogenicity along with hERG Inhibition

Name of the compound	Mutagenicity	Toxicity Carcinogenicity		hERG inhibition
		Rat	Mouse	
Naringenin	Mutagen	Positive	Negative	Medium risk
Hesperidin	Non mutagen	Negative	Negative	High risk
Apigenin	Mutagen	Positive	Negative	Medium risk
Luteolin	Mutagen	Positive	Negative	Medium risk
Chalcone	Mutagen	Negative	Negative	Medium risk

Table 4: Table Showing Results of Oral Rat Acute Toxicity and Maximum Tolerated Dose for Human

Name of the compound	Oral Rat Acute Toxicity (LD50) (mol/kg)	Max. tolerated dose (human)(log mg/kg/day)
Naringenin	1.791	-0.176
Hesperidin	2.506	0.525
Apigenin	2.45	0.328
Luteolin	2.455	0.499
Chalcone	1.843	1.031

Bioactivity Score Prediction Using Molinspiration Chemoinformatics Tool

Table 5 shows the calculated scores predicted by the Molinspiration chemoinformatics online tool for the various bioactivities of the substances. The bioactivity scores for each substance vary from 0 to -5.0, indicating the different levels of activity[68].

Table 5: Table Showing Results of Bioactivity Score Prediction

Name of the compound	Bioactivity					
	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
Naringenin	0.03	-0.20	-0.26	0.42	-0.12	0.21
Hesperidin	-0.01	-0.59	-0.36	-0.20	-0.00	0.06
Apigenin	-0.07	-0.09	0.18	0.34	-0.25	0.26
Luteolin	-0.02	-0.07	0.26	0.39	-0.22	0.28
Chalcone	-0.43	-0.18	-0.66	-0.51	-0.60	-0.12

Molecular Docking Interaction Using AutoDock Vina

Based on the docking analysis done by AutoDock Vina, binding affinity of different *Citrus* compounds with the relevant protein Human Aromatase Cytochrome p450 is determined. Ligands showing more negative binding energy actually exhibit the highest binding affinity towards the proteins. According to our study, we have selected five ligands, each ligand shows different result as their binding capacity with the target protein receptors were different. As reported by the docking result, hesperidin shows the maximum binding affinity i.e. -9.7 kcal/ mol. followed by luteolin (-8.3 kcal/mol.), apigenin (-8 kcal/mol), naringenin (-7.8 kcal/mol) and lastly it is chalcone that shows the minimum affinity energy -7.4 kcal/mol. The total result of different binding energy affinity is represented in Fig. 7 and each of the docking interactions is presented both in 3D and 2D manner (Figure 8A1-8A2 and 8B1-8B2).

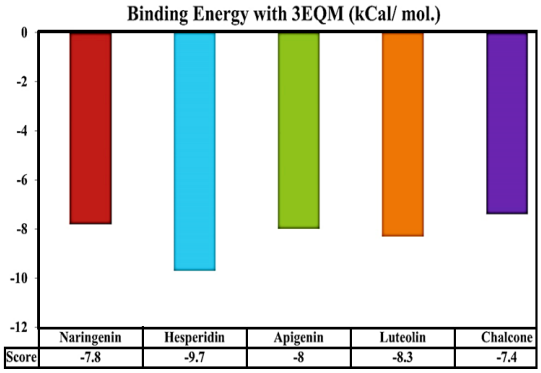

Fig. 7. Graphical Representation of Binding Affinity along with the Score (kCal/mol.) towards the Target Protein 3EQM by the Respective Compounds/Ligands

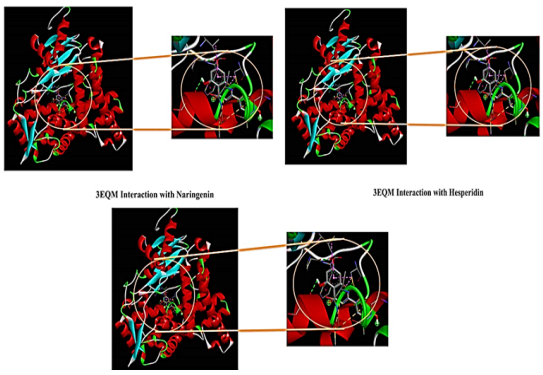

Fig. 8A1. Complete Representation and Close Insight of 3EQM Interaction with the Respective Three Ligands

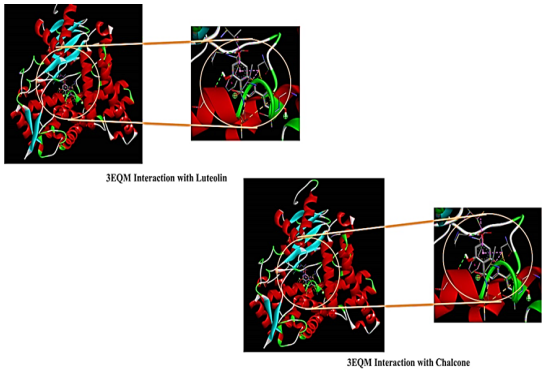

Fig. 8A2. Complete Representation and Close Insight of 3EQM Interaction with the Respective Two Ligands

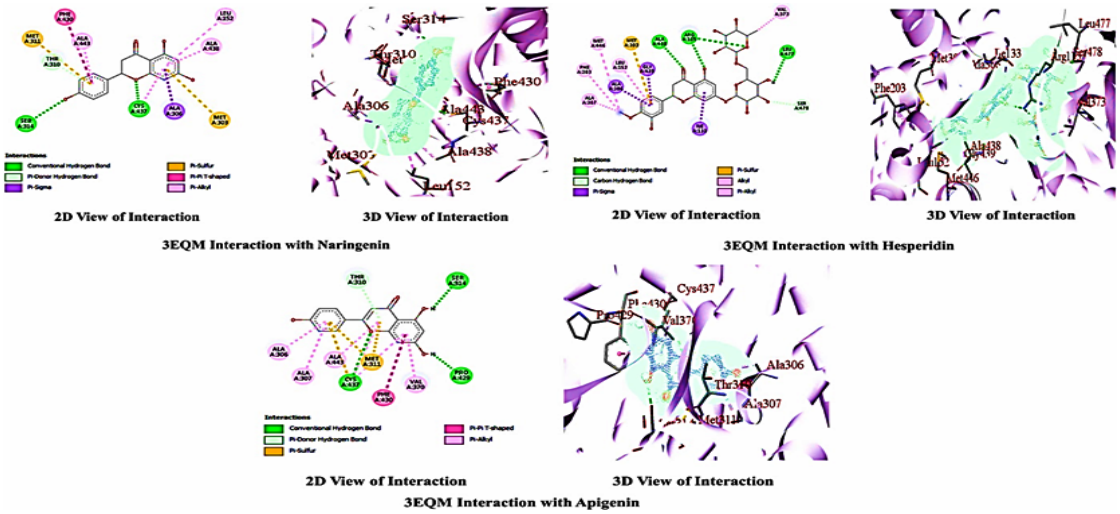

Fig. 8B1. 2D and 3D View of 3EQM Interaction with the Respective Three Ligands

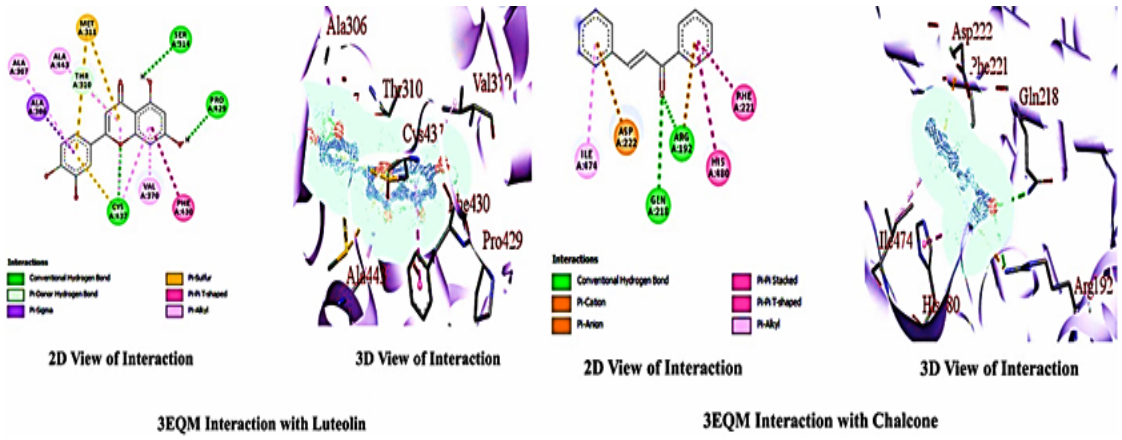

Fig. 8B2. 2D and 3D View of 3EQM Interaction with the Respective Two Ligands

Assessment of Active Amino Acid Residues or Structural Hotspots on the Receptor Protein

Table 6 displays the findings from the CASTp 3.0 web server for various protein PDB structures. The key amino acids implicated in the particular protein-ligand interaction are displayed in this finding.

Table 6: Table Showing Active Amino Acid Residues Obtained Via Molecular Docking Interaction for Each of the Ligand Along with the Receptor Protein 3EQM

Name of the protein	Name of the Compound/Ligand	Active Amino Acid Residues
3EQM	Naringenin	LEU 152, MET 303, ALA 306, THR 310, MET 311, SER 314, PHE 430, CYS 437, ALA 438, ALA 443
	Hespereidin	ARG 115, ILE 133, LEU 152, PHE 203, MET 303, ALA 306, ALA 307, VAL 373, ALA 438, GLY 439, MET 446, LEU 477, SER 478
	Apigenin	ALA 306, ALA 307, THR 310, MET 311, SER 314, VAL 370, PRO 429, PHE 430, CYS 437, ALA 443
	Luteolin	ALA 306, ALA 307, THR 310, MET 311, SER 314, VAL 370, PRO 429, PHE 430, CYS 437, ALA 443
	Chalcone	ARG 192, GLN 218, PHE 221, ASP 222, ILE 474, HIS 480

iMod Server Prediction

iMod Server prediction represents normal mode analysis in internal coordinates, generally shows results in the form of different factor analysis have been shown in Fig. 9A-9B. iMod interaction status between protein-ligand docked structure, here the black coloured arrows show the mode of interaction between the receptor-ligand; in addition of the interaction status, Deformability, experimental B Factor, Eigenvalue, covariance Map and well as Elastic Network defines represent the overall protein ligand interaction mode.

CABS-flex 2.0 Server prediction

The MD-simulation study on CABS-flex 2.0 webserver was carried out, from where we basically derived the RMSF plots of each of the docked complexes, is represented in a pictorial manner Fig. 10A-10B. Here in this graph almost different pattern of fluctuation state of the amino acids involved in the interaction between Human Aromatase Cytochrome p450 Protein and small molecules/ ligands is observed.

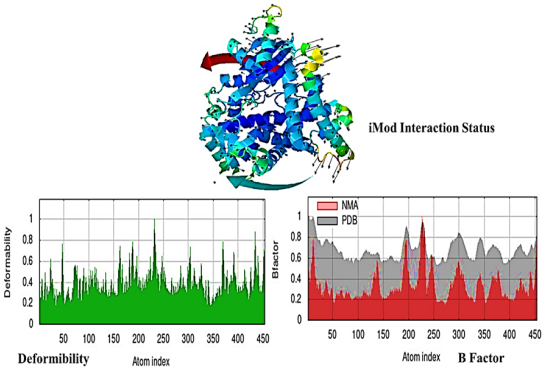

Fig. 9A. Results of iMod Server Prediction of 3EQM-Ligand Complex

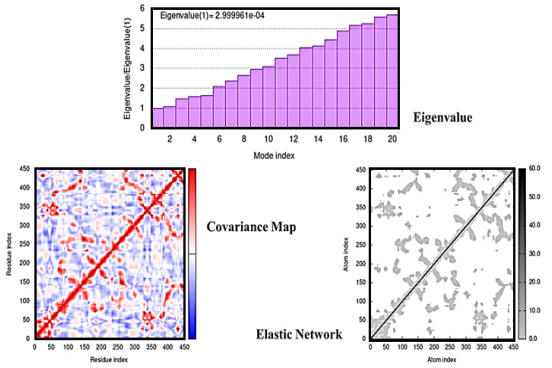

Fig. 9B. Results of iMod Server Prediction of 3EQM-Ligand Complex

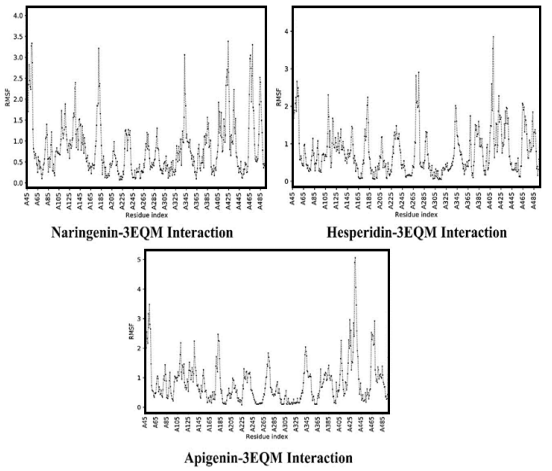

Fig. 10A. Results of CABS-flex 2.0 Server Prediction of 3EQM with their respective ligand Complex

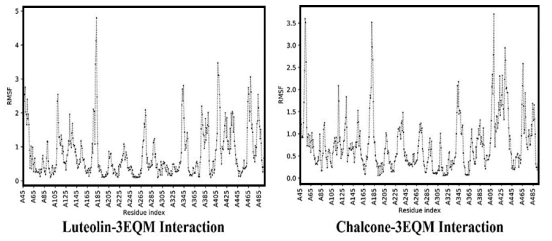

Fig. 10B. Results of CABS-flex 2.0 Server Prediction of 3EQM with their respective ligand Complex

CONCLUSION

In this study, one of the major classes of protein i.e. Human Aromatase Cytochrome p450 was selected on the basis of *Citrus* species bioactive compounds mediated receptor target prediction. This enzyme plays the decisive part in oestrogen biosynthesis, mainly associated in the alteration of androgens to oestrogens. For being play a pivotal role in the expansion of breast cancer, endocrine based remedy by blocking aromatase enzyme should be our focus of thinking concerning the inhibition of further chances of breast cancer progression. Seeing the aromatase inhibitory activities of *Citrus* species natural phyto-compounds, five major phytochemicals i.e. Naringenin, Hesperidin, Apigenin, Luteolin and Chalcone have been selected on the basis of their therapeutic potency. All of these five ligands, show active target prediction towards the aromatase group of enzyme. When the *Citrus* bioactive compounds were analysed by *in silico* computational docking tools (AutoDock Vina), they were successfully docked and showed great binding energy against the specific protein/receptor. Depending on their binding affinity, they are categorized under degree of Naturel inhibitors against human aromatases enzyme. Additionally, it was noted that the majority of the compounds have favourable physicochemical profiles and a number of other ADMET features, such as drug-like property predictions that revealed that all but hesperidin fulfil Ro5. Additionally, each substance did exhibit high to low chances of blocking hERG. Furthermore, *In-vitro* and *In-vivo* toxicological investigations should be used to confirm these prediction results.

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Conflict of interest

The authors affirm that there are no conflicts of interest.

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