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Research Article

IDENTIFICATION OF POTENTIAL COX-2 INHIBITORS FROM PHYTOCHEMICAL CONSTITUENTS OF INDIAN "GARAM MASALA" USING *IN SILICO* ANALYSIS

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ABSTRACT

Objective: The objective of the study was to analyze the active principles of "Garam Masala" (routinely used spice-mix in Indian cuisine) for their anti-inflammatory potential against Cyclooxygenase-2 (COX-2), a crucial player in inflammatory response in humans, using molecular docking simulation.

Methods: After obtaining three-dimensional structures of spice phytochemicals and COX-2 protein from PUBCHEM and PDB databases, phytochemicals with suitable absorption, distribution, metabolism, and excretion (ADME) properties were docked against COX-2 protein using PyRx and AutoDock tools 1.5.6 and their binding properties were compared with "Coxibs" drugs (NSAIDs, known COX-2 inhibitors) to establish their anti-COX-2 potential.

Results: Farnesiferol A showed better binding affinity to COX-2 whereas three other phytochemicals Piperine, Cedrelanol, and Usnic acid demonstrated comparable binding affinity like those of "coxibs."

Conclusion: Molecular docking simulation and ADME analysis reveal that Farnesiferol A, Piperine, Cedrelanol, and Usnic acid could be considered for potential drug candidates for COX-2 inhibition due to their promising binding affinities with COX-2.

Keywords: Cyclooxygenase, Coxibs, phytochemicals, NSAIDs, COX-2 inhibitor, Molecular docking.

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INTRODUCTION

Inflammation, a generic response of a body against harmful stimuli, is a necessary evil. It is considered to be a part of innate immune response against detrimental factors such as pathogens, damaged cells or tissues and irritants. The main purpose is to eradicate the pathogens or pathogenic signatures, that is, pathogen-associated molecular pattern molecules or any cause of cellular injury; or to eliminate damaged cells, tissues, and associated molecular signatures, that is, damage-associated molecular pattern molecules and initiate tissue repairing procedures. On one side, inflammation at a very low level could lead to progressive cellular damage and also might compromise with the survival of the organism; on the other hand, chronic or uncontrolled inflammation leads to various disorders such as allergy, atherosclerosis, arthritis, hay fever, and cancer.

Cyclooxygenases (COX), the crucial mediators of inflammatory processes, are also called prostaglandin G/H synthases which regulate the bis-dioxygenation and subsequent reduction of arachidonic acid to prostaglandins. Prostaglandins are involved in various patho-physiological processes such as pain, fever, inflammation, thrombosis, gastrointestinal cytoprotection, and tumorigenesis [1]. Two isoforms of COX (COX 1 and COX2) are thoroughly studied and are considered to be the potential targets of widely used nonsteroidal anti-inflammatory drugs (NSAIDs). COX-1 is ubiquitously expressed whereas COX-2 expression is subjected to immunogenic stimuli and therefore is a better anti-inflammatory drug target [2-4]. Prostanoids, which are derived from COX-2, have many constructive roles as well, such as maintaining vaso-bronchial health, inhibiting platelet aggregation, vasodilation, and regulating contraction of smooth muscle tissue [5]. This dual role of COX-2 activity makes it particularly difficult to design an anti-inflammatory drug based on COX-2 inhibitions. "Coxibs" (such as Celecoxib and Rofecoxib) are generally used as COX-2 inhibitors but these drugs also impart certain side effects inside our body such as increased clotting and subsequent heart attacks [5].

Human COX-2 enzyme constitutes of two identical chains (homo-dimer) of 581 amino acids and four oligosaccharides [6]. Oligosaccharides are structurally significant as they regulate the folding mechanism. Three high mannose oligosaccharides are responsible for proper folding and the fourth one regulates its degradation [6]. To elucidate the tertiary structure further, each dimer consists of functionally exclusive domains - epidermal growth factor domain, membrane binding domain, and a catalytic domain. The catalytic domain is responsible for COX and peroxidises activity [6]. COX-1 and COX-2 are almost identical in their tertiary structures with certain minor differences [7]. Three COX-1 COX channel residues Ile-434, His-513, and Ile-523 are substituted with Val-434, Arg-513, and Val-523, respectively, in COX-2. These substitutions resulted in certain significant changes in COX-2 such as increased volume of its active sites and generation of a side pocket with Arg-513 located at the base of it (Garavito *et al*., 2002). The coxibs were initially designed to bind inside the side pocket to provide isoform-selective inhibition. Substitution of Ile with Val at the 523rd position of COX-2 enhances the inhibition even further. X ray crystallographic analysis of Vioxx (Rofecoxib) and Celecoxib bound with human COX-2 enzyme reveals that binding of both inhibitors is conserved with the sulfone (Vioxx) or sulfonamide (Celecoxib) moiety inserted into side pocket near Arg-513 [7]. Tyr-385 also plays an important role in the active site [8]. Spices are long being used in combating chronic inflammatory diseases such as arthritis, cardiovascular diseases, cancer, neurodegenerative disease, and asthma. A spice is any part of the plant (root, seed, bark, berry, bark, and parts of flower) or any plant substance that is used as a flavoring agent in culinary practices. Apart from its uses in food, spices are of tremendous medicinal importance, containing a battery of medicinally significant phytochemicals. "Garam masala" or spice mix is an amalgamation of different spices (mainly of South Asian origin) used commonly in Indian cuisine, alone or as a seasoning agent to enhance the overall taste of food. The composition of the blend is subjected to regional diversity and there is no one more authentic than others [9]. A quintessential Indian "Garam masala" contains black pepper, cloves,

Table 1: Extracted protein information

Protein ID	Macromolecule	Method	Organism	Unique ligands	Unique branched mono saccharides
5KIR	Prostaglandin G/H synthase 2 (COX-2)	X-ray diffraction	*Homo sapiens*	Protoporphyrin IX containing CO Rofecoxib 2-acetamido-2 -deoxy-beta-D-glucopyranose Phosphate ion Glycerol Ammonium ion	MAN (Mannose) NAG (2-acetamido-2 -deoxy-beta-D-glucopyranose)

Table 2: Spices and phytochemical components of Indian "garam masala"

Spices	Plant source (Scientific names)	Phytochemical constituents
Black pepper	*Piper nigrum* L.	Piperine, β-caryophyllene, limonene, δ-3-carene, α-pinene, β-pinene, α-phellandrene, myrcene, terpinolene
Red pepper	*Capsicum annuum* L.	Capsaicin, β-carotene, zeaxanthin, lutein, cafeic acid, capsanthin
Cardamom	*Elettaria cardamomum* (L.) Maton	1,8-cineole, α-terpinyl acetate, limonene, linalool, terpinolene, myrcene, linalyl acetate
Cinnamon	*Cinnamomum verum* J.Presl	Cinnamaldehyde, cinnamyl acetate, cineole, eugenol, coumarin, linalool, humulene, ethyl cinnamate, β-caryophyllene, cedrelanol
Clove	*Syzygium aromaticum* (L.) Merr. & L.M. Perry	Eugenol, eugenyl acetate, α-humulene, β-caryophyllene
Black cumin	*Nigella sativa* L.	Thymoquinone, cuminaldehyde, γ-terpinene, β-pinene, p-cymene, p-mentha-1,3-diene-7-al, p-mentha-1,4-dien-7-al
Star anise	*Illicium verum* Hook.f.	Estragole, aretrans-anethole, limonene, phenylpropanoids
Fennel	*Foeniculum vulgare* Mill.	Estragole, trans-anethole, fenchone, limonene, anisaldehyde, sabinene, β-myrcene, α-pinene, β-pinene, camphene
Coriander	*Coriandrum sativum* L.	Petroselinic acid, linoleic acid, oleic acid, palmitic acid, stearic acid, vaccenic acid, myristic acid
Nutmeg/ mace	*Myristica fragrans* Houtt.	Eugenol, methyleugenol, methylisoeugenol, elemicin, myristicin, safrole
Bay leaves	*Laurus nobilis* L.	1,8-cineole, α-pinene, limonene, alpha-terpinyl acetate, terpinene-4-ol
Asafoetida	*Ferula assa-foetida* L.	Ferulic acid, umbel-liferone, asaresinotannols, farnesiferols A, B, C, glucose, galactose, l-arabinose, rhamnose, glucuronic acid, 2-butyl propenyl disulfde
Stone flower	*Parmotrema perlatum* (Huds.) M.Choisy	Usnic scid, Lecanoric acid, Atranorin

cinnamon, mace, fennel, cardamoms, cumin, red chili, coriander seeds, and fennel; however, some recipes also include star anise, asafetida, and stone flower [10]. In general, the spices are roasted and properly blend before use to utilize its flavors and aroma maximally. Besides its seasoning and aromatic property, all the ingredients are also reservoirs of medicinally active compounds, which are useful against chronic inflammations. The natural compounds could be beneficial against inflammation targets as an alternative of modern NSAIDs which result in lot of side effects. Here we have tried to virtually analyze the anti-inflammatory potential of selected phytochemicals from all-inclusive Indian "Garam Masala" using molecular docking approach.

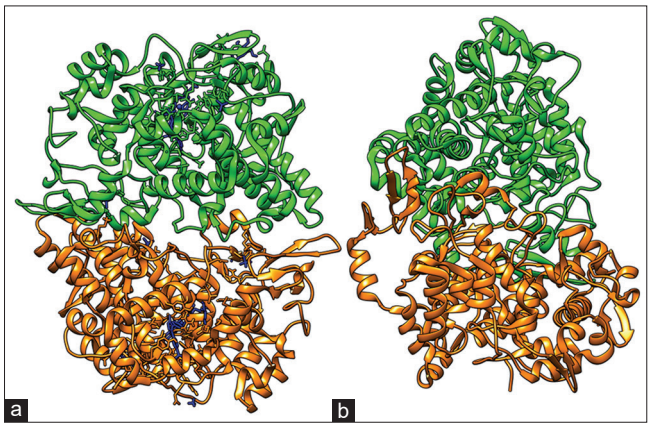

Fig. 1: Protein structure (5KIR) visualized with UCSF CHIMERA 1.15 a. Structure of COX-2 with associated ligands. b. Structure of COX-2 after cleaning. (Orange: Chain A; Green: Chain B; Blue: Associated ligands)

METHODS

Preparation of target protein

COX-2 protein file was obtained from RCSB PDB server (PDB ID: 5KIR) [11] and cleaned using UCSF CHIMERA1.15 for docking studies. Stereo-chemical properties were analyzed by preparing Ramachandran plot using Z lab [12] and PROCHEK server [13].

Selection of phytochemicals as potential ligands

Spice components, plant source, and active compounds of Indian "garam masala" were fetched from IMPPAT [14] and WORLD FLORA ONLINE database [15] after reviewing different literatures [16]. At first, a library of 36 phytoconstituents was prepared for virtual screening to find out the potential ligands. 3D structures (Fig. 1) of all the compounds were obtained from PUBCHEM database in SDF formats and converted into PDB formats using PYMOL2.5.1. Physio-chemical properties were picked up from PUBCHEM open chemistry database. Two known inhibitors of COX-2, namely, Rofecoxib and Celecoxib were used as controls in this experiment.

Analysis of ligand suitability as drug

The *drug likeness* of the potential ligands was evaluated using *Lipinski filter* which is based on *Lipinski's Rule of five* that determines the "drugability" of a chemical compound to be used as an orally active drug in humans [17]. A compound must qualify on the grounds of certain pharmacokinetic properties, namely, molecular weight, lipophilicity,

Table 3: Physio-chemical properties of potential ligands

Ligands	PUBCHEM ID	Molecular formula	Molecular weight	Heavy atom count	Topological polar surface area (Å)
Piperine	CID638024	$C_{17}H_{19}NO_3$	285.34	21	38.77
Beta caryophylline	CID5281515	$C_{15}H_{24}$	204.35	15	0
Limonene	CID22311	$C_{10}H_{16}$	136.23	10	0
Alpha-pinene	CID6654	$C_{10}H_{16}$	136.23	10	0
Alpha-phellandrene	CID7460	$C_{10}H_{16}$	136.23	10	0
Myrcene	CID31253	$C_{10}H_{16}$	136.23	10	0
Terpinolene	CID11463	$C_{10}H_{16}$	136.23	10	0
Capsaicin	CID1548943	$C_{18}H_{27}NO_3$	305.41	22	58.56
Beta carotene	CID	$C_{40}H_{56}$	536.87	40	0
Eucalyptol	CID2758	$C_{10}H_{18}O$	154.25	11	9.23
Alpha terpinyl acetate	CID111037	$C_{12}H_{20}O_2$	196.29	14	26.3
Linalool	CID6549	$C_{10}H_{18}O$	154.25	11	20.23
Linalyl acetate	CID8294	$C_{12}H_{20}O_2$	196.29	14	26.3
Cinnamaldehyde	CID637511	C_9H_8O	132.16	10	17.07
Eugenol	CID3314	$C_{10}H_{12}O_2$	164.2	12	29.46
Coumarin	CID323	$C_9H_6O_2$	146.14	11	30.21
Cedrelanol	CID160799	$C_{15}H_{26}O$	222.37	16	20.23
Thymoquinone	CID10281	$C_{10}H_{12}O_2$	164.2	12	34.14
Cuminaldehyde	CID326	$C_{10}H_{12}O$	148.2	11	17.07
Gamma-terpinene	CID7461	$C_{10}H_{16}$	136.23	10	0
Estragole	CID8815	$C_{10}H_{12}O$	148.2	11	9.23
Trans-anethole oxide	CID10080713	$C_{10}H_{12}O_2$	164.2	12	21.76
Fenchone	CID14525	$C_{10}H_{12}O$	148.2	11	9.23
Anisaldehyde	CID31244	$C_8H_8O_2$	136.15	10	26.3
Elemicin	CID10248	$C_{12}H_{16}O_3$	208.25	15	27.69
Myristicin	CID4276	$C_{11}H_{12}O_3$	192.21	14	27.69
Safrole	CID5144	$C_{10}H_{10}O_2$	162.19	12	18.46
Ferulic acid	CID445858	$C_{10}H_{10}O_4$	194.18	14	66.76
Umbelliferone	CID5281426	$C_9H_6O_3$	162.14	12	50.44
Farnesiferol A	CID7067262	$C_{24}H_{30}O_4$	382.49	28	59.67
Lecanoric acid	CID99613	$C_{16}H_{14}O_7$	318.28	23	124.29
Atranorin	CID68066	$C_{19}H_{18}O_8$	374.34	27	130.36
Capsanthin	CID5281228	$C_{40}H_{56}O_3$	584.87	43	57.53
Lutein	CID5281243	$C_{40}H_{56}O_2$	568.87	42	40.46
Zeaxanthin	CID5280899	$C_{40}H_{56}O_2$	568.87	42	40.46
Usnic acid	CID5646	$C_{18}H_{16}O_7$	344.32	25	117.97
Known ligands					
Rofecoxib	CID5090	$C_{17}H_{14}O_4S$	314.4	22	68.8
Celecoxib	CID2662	$C_{17}H_{14}F_3N_3O_2S$	381.4	26	86.4

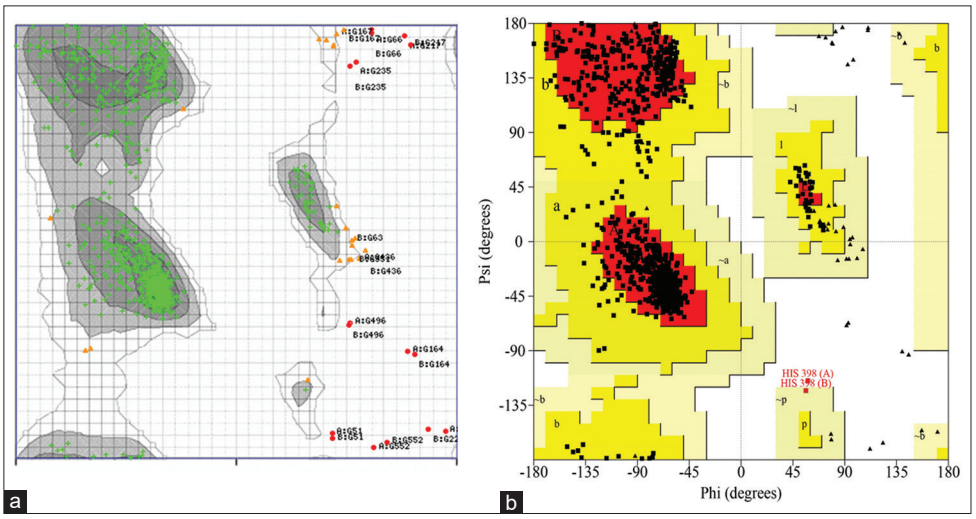

Fig. 2: Stereo-chemical property analysis using Ramachandran plot. (a) Using Z Lab server [2]. Black, Dark grey, Grey, and Light grey represent highly preferred conformations (Delta ≥–2). White with Black grids represent preferred conformations (–2> Delta ≥–4). White with Grey grid represents questionable conformations (Delta <–4). Highly preferred observations (96.633%) shown as GREEN crosses, preferred observations (1.911%) shown as BROWN triangles as questionable observations shown as RED circles (1.456%). (b) Plot statistics analysis using PROCHEK server [15]

polar surface area, hydrogen bonding, and charge; as stated within the rule; before using it as a drug in the human system. It also involves its absorption, distribution, metabolism, and excretion (ADME) in the human body.

Table 4: Lipinski filter analysis

Ligands	Molecular formula	Molecular weight	H-bond donors	H-bond acceptors	MLOGP	Lipinski violations
Piperine	$C_{17}H_{19}NO_3$	285.34	0	3	2.39	0
Beta caryophylline	$C_{15}H_{24}$	204.35	0	0	4.63	1
Limonene	$C_{10}H_{16}$	136.23	0	0	3.27	0
Alpha-pinene	$C_{10}H_{16}$	136.23	0	0	4.29	1
Alpha-phellandrene	$C_{10}H_{16}$	136.23	0	0	3.27	0
Myrcene	$C_{10}H_{16}$	136.23	0	0	3.56	0
Terpinolene	$C_{10}H_{16}$	136.23	0	0	3.27	0
Capsaicin	$C_{18}H_{27}NO_3$	305.41	2	3	2.69	0
Beta carotene	$C_{40}H_{56}$	536.87	0	0	8.96	2
Eucalyptol	$C_{10}H_{18}O$	154.25	0	1	2.45	0
Alpha terpinyl acetate	$C_{12}H_{20}O_2$	196.29	0	2	2.65	0
Linalool	$C_{10}H_{18}O$	154.25	1	1	2.59	0
Linalyl acetate	$C_{12}H_{20}O_2$	196.29	0	2	2.95	0
Cinnamaldehyde	C_9H_8O	132.16	0	1	2.01	0
Eugenol	$C_{10}H_{12}O_2$	164.2	1	2	2.01	0
Coumarin	$C_9H_6O_2$	146.14	0	2	1.65	0
Cedrelanol	$C_{15}H_{26}O$	222.37	1	1	3.67	0
Thymoquinone	$C_{10}H_{12}O_2$	164.2	0	2	1.08	0
Cuminaldehyde	$C_{10}H_{12}O$	148.2	0	1	2.4	0
Gamma-terpinene	$C_{10}H_{16}$	136.23	0	0	3.27	0
Estragole	$C_{10}H_{12}O$	148.2	0	1	2.67	0
Trans-anethole oxide	$C_{10}H_{12}O_2$	164.2	0	2	1.44	0
Fenchone	$C_{10}H_{12}O$	148.2	0	1	2.67	0
Anisaldehyde	$C_8H_8O_2$	136.15	0	2	1.12	0
Elemicin	$C_{12}H_{16}O_3$	208.25	0	3	1.97	0
Myristicin	$C_{11}H_{12}O_3$	192.21	0	3	1.7	0
Safrole	$C_{10}H_{10}O_2$	162.19	0	2	2.02	0
Ferulic acid	$C_{10}H_{10}O_4$	194.18	2	4	1	0
Umbelliferone	$C_9H_6O_3$	162.14	1	3	1.04	0
Farnesiferol a	$C_{24}H_{30}O_4$	382.49	1	4	3.83	0
Lecanoric acid	$C_{16}H_{14}O_7$	318.28	4	7	1.55	0
Atranorin	$C_{19}H_{18}O_8$	374.34	3	8	1.4	0
Capsanthin	$C_{40}H_{56}O_3$	584.87	2	3	6.08	2
Lutein	$C_{40}H_{56}O_2$	568.87	2	2	6.96	2
zeaxanthin	$C_{40}H_{56}O_2$	568.87	2	2	6.96	2
usnic acid	$C_{18}H_{16}O_7$	344.32	2	7	-0.52	0
Known inhibitors						
Rofecoxib	$C_{17}H_{1404}S$	314.4	0	4	2.62	0
Celecoxib	$C_{17}H_{14}F_3N_{302}S$	381.4	1	7	2.65	0

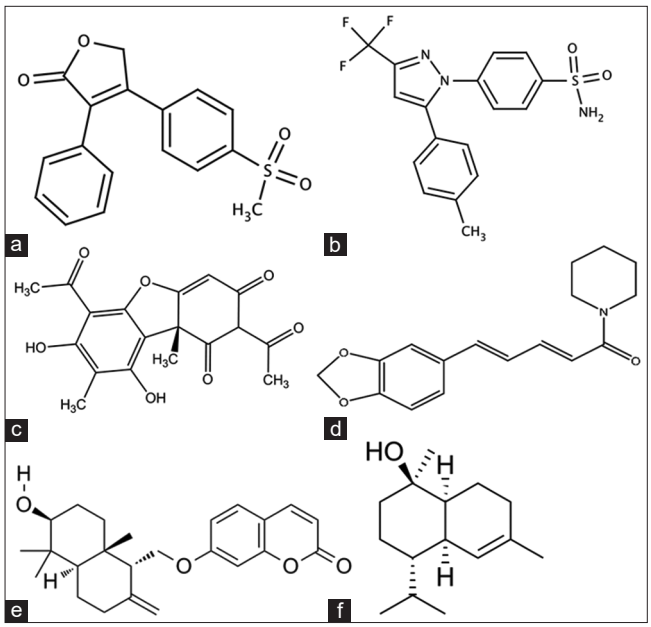

Fig. 3: Molecular structure of selected compounds. (a) Rofecoxib, (b) Celecoxib, (c) Usnic acid, (d) Piperine, (e) Farnesiferol A, and (f) Cedrelanol

These 36 phytoconstituents were subjected to Lipinski filter and ADMESAR analysis to examine their drug likeness. Molecules with less reasonable stereo chemical properties were discarded after this step. Criteria used for Lipinski filter analysis were molecular weight ≤500, hydrogen bond donor ≤5, hydrogen bond acceptor ≤10, and octanol water partition coefficient (LogP) ≤5. ADME properties were also examined using SWISS ADME; a conventional drug discovery tool to evaluate drug likelihood, pharmacokinetics, and medicinal chemistry friendliness of small compounds [17].

Screening of ligands for molecular docking

Compounds selected from SWISS ADME analysis were screened further according to their binding energies against the COX-2 protein target using PyRx; which is a virtual screening software used in computational drug discovery [17]. Selected compounds were also analyzed for their permeability to blood–brain barrier and gastrointestinal absorption using Brain Or Intestinal Estimated Permeation Method (BOILED-Egg) [17,18] before docking.

BOILED-Egg is an intuitive way to evaluate two key ADME parameters-passive gastrointestinal absorption (HIA) and brain penetration (BBB) as a function of two physiochemical descriptors WLOGP and TPSA (for lipophilicity and apparent polarity). The egg shaped plot comprises of the *yolk* signifying the physiochemical space denoting high probability of BBB permeation whereas the *white* portion signifies the physiochemical space for highly probable HIA absorption. Outside

Table 5: ADMESAR analysis

Ligands	GI absorption	BBB permeant	PGP substrate	ALI LOG S	ALI class
Piperine	High	Yes	No	−3.96	Soluble
Beta caryophylline	Low	No	No	−4.1	Moderately soluble
Limonene	Low	Yes	No	−4.29	Moderately soluble
Alpha-pinene	Low	Yes	No	−4.2	Moderately soluble
Alpha-phellandrene	Low	Yes	No	−2.88	Soluble
Myrcene	Low	Yes	No	−3.88	Soluble
Terpinolene	Low	Yes	No	−4.19	Moderately soluble
Capsaicin	High	Yes	No	−4.5	Moderately soluble
Beta carotene	Low	No	Yes	−13.6	Insoluble
Eucalyptol	High	Yes	No	−2.59	Soluble
Alpha terpinyl acetate	High	Yes	No	−4.21	Moderately soluble
Linalool	High	Yes	No	−3.06	Soluble
Linalyl acetate	High	Yes	No	−4.18	Moderately soluble
Cinnamaldehyde	High	Yes	No	−1.88	Very soluble
Eugenol	High	Yes	No	−2.53	Soluble
Coumarin	High	Yes	No	−1.63	Very soluble
Cedrelanol	High	Yes	No	−3.44	Soluble
Thymoquinone	High	Yes	No	−2.55	Soluble
Cuminaldehyde	High	Yes	No	−2.37	Soluble
Gamma-terpinene	Low	Yes	No	−4.22	Moderately soluble
Estragole	High	Yes	No	−3.24	Soluble
Trans-anethole oxide	High	Yes	No	−1.87	Very soluble
Fenchone	High	Yes	No	−3.24	Soluble
Anisaldehyde	High	Yes	No	−1.93	Very soluble
Elemicin	High	Yes	No	−2.76	Soluble
Myristicin	High	Yes	No	−3.18	Soluble
Safrole	High	Yes	No	−3.01	Soluble
Ferulic acid	High	Yes	No	−2.52	Soluble
Umbelliferone	High	Yes	No	−2.25	Soluble
Farnesiferol a	High	Yes	Yes	−5.94	Moderately soluble
Lecanoric acid	High	No	No	−5.93	Moderately soluble
Atranorin	High	No	No	−6.78	Poorly soluble
Capsanthin	Low	No	Yes	−11.76	Insoluble
Lutein	Low	No	Yes	−11.83	Insoluble
Zeaxanthin	Low	No	Yes	−11.72	Insoluble
Usnic acid	High	No	No	−5.02	Moderately soluble
Known inhibitors					
Rofecoxib	High	Yes	No	−3.35	Soluble
Celecoxib	High	No	No	−4.89	Moderately soluble

grey region is for the molecules with limited blood brain barrier permeability and predicted low gastrointestinal absorption. The points are labeled in blue if predicted to be actively effluxed by P glycoprotein (PGP+) and red if predicted to be a non-substrate for P glycoprotein (PGP-) [17,18].

Molecular docking studies

Molecular docking study was performed to predict the interaction of the selected phytochemicals with COX-2 receptor protein target using AutoDock tools 1.5.6 software. Experiments were carried out with default parameters to get the accurate result. Entire docking process was performed on a Windows10 workstation with AMD Quad- core processor (A10-9600P RADEON R5, 2.40 GHz) and 4 GB RAM.

An extended PDB format; PDBQT files for ligands and protein which include atomic partial charges and atom types, grid box, and grid parameter files were generated using Graphical User Interface of AutoDock Tools. Protein was prepared by deleting water molecules, assigning polar hydrogens, Kollman charges, fragmental volumes, and salvation parameters and saved in PDBQT format [19]. Autogrid procedure was performed for generating the grid map by embedding the protein into a three-dimensional grid box. The grid size was set to 84×112×108 xyz points with grid spacing of 0.503 Å and grid center was designated at dimensions (x, y, and z): −1.444, −3.694, and −4.778. Grid box was designed based on important amino acid residues of the active site such as Val-344, Trp-387, Phe-518, Arg-513, Glu-524, His-90, Tyr-355, Arg-120, Leu-531, Ser-530, Val-523, and Tyr-385 [7]. For each type of atom in the ligands, AutoDock affinity grids are measured along with electrostatic and desolvation potentials. Later on, the energetics of a particular ligand configuration are evaluated using the values from the grids using AutoDock calculation. Docking was carried out using Lamarckian genetic algorithm with 10 runs and the most favorable configuration was selected from cluster RMSD table. The conformations with lowest binding energies were extracted from the DLG files and aligned with protein molecule for further analysis.

Analysis of docking results

The conformation with the lowest binding energy of each ligand was extracted from DLG files and analyzed on AutoDock tools. Protein-ligand docked complexes were saved in PDBQT format and subsequently converted into PDB format using OpenBabel 2.3.1. Next, the complexes were visualized on CHIMERA 1.15. Receptor ligand interaction was further analyzed using DISCOVERY STUDIO VISUALIZER (2021) in both 2D and 3D formats. Bond statistics involved in receptor ligand interaction were also analyzed in depth on Protein-Ligand interaction profiler (PLIP) server [20] an on Proteinplus server [21] and visualized on PymoL 2.5.1.

RESULTS AND DISCUSSION

Pre docking procedures

Protein file preparation

Information of COX-2 protein PDB file (PDB ID: 5KIR) is mentioned in Table 1 and the Ramachandran plots showing stereo-chemical properties are shown in Fig. 1.

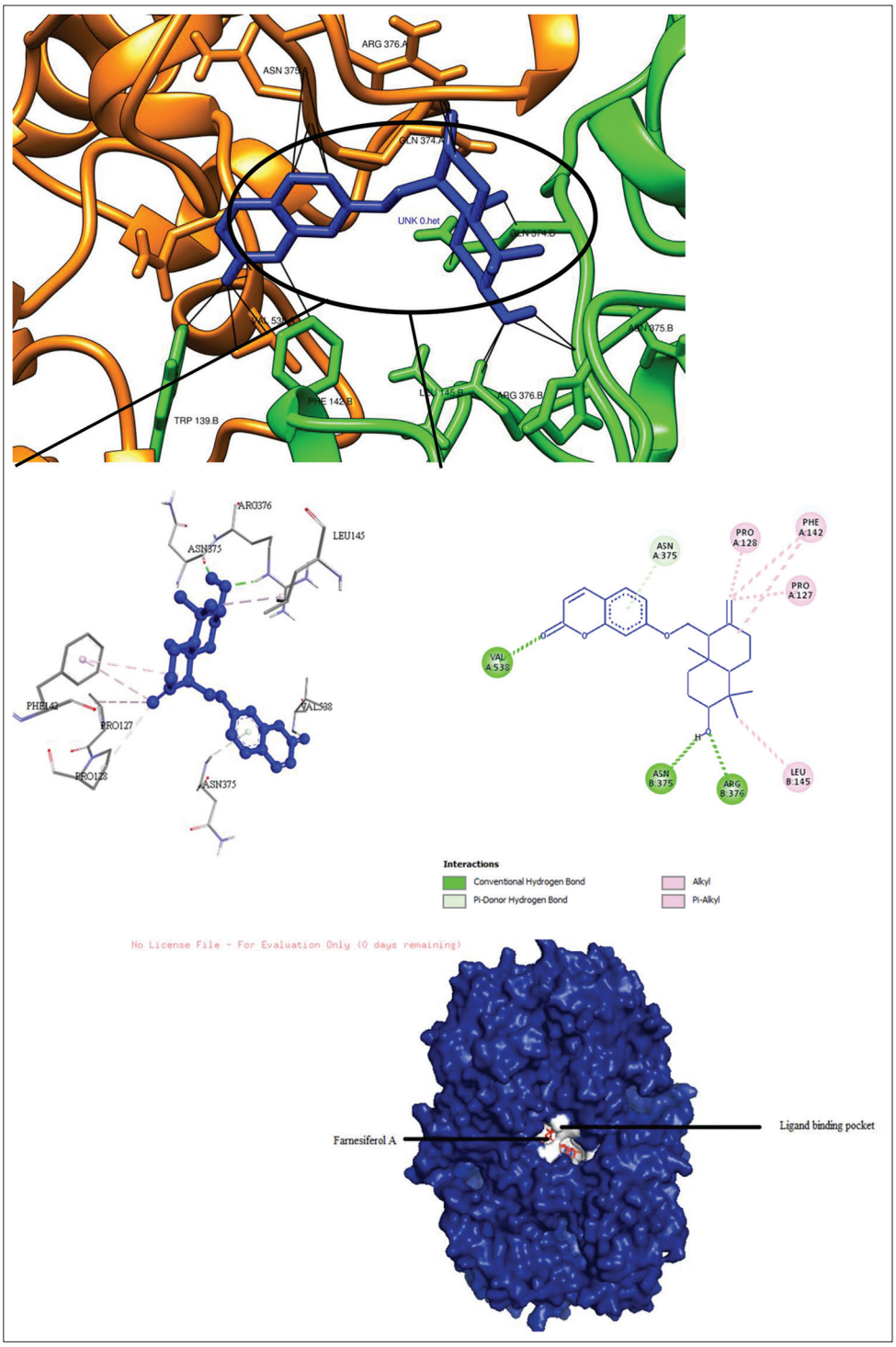

Fig. 4: Receptor-Ligand interaction of Farnesiferol A with COX-2 binding site

Total number of residues observed-1102, out of which 841 residues lie in most favored region (A, B, and L) mentioned in Fig 4, 101 in additional allowed region (a, b, l, and p), 1 in generously allowed region (~a, ~b, ~l, and ~p), and 1 in disallowed region. Number of Glycine residues (shown as triangles) are found to be 72 and proline residues 76, non-glycine non-proline residues are 952, and end-residues excluding glycine and proline are 2.

Ligand library generation

Source and phytoconstituents of Indian "garam masala" are listed in Table 2.

Physio-chemical properties of all 36 compounds, obtained from PUBCHEM open chemistry database, are listed in Table 3.

Analysis of drug likeness

The phytoconstituents were subjected to Lipinski filter and ADMESAR analysis for the first layer of screening. Molecules with less reasonable stereo chemical properties were discarded after this step. The results are summarized in Tables 4 and 5.

Compounds with no more than 1 Lipinski violation were then selected for further screening.

Screening of ligands using PyRx

Phytochemicals which were selected after Lipinski filter analysis were further screened based on their binding energies with COX-2 receptor protein using PyRx and presented in Table 6.

Finally, 10 compounds showing binding energy less than -7kcal/mol and no more than 1 Lipinski violations (Farnesiferol A, Atranorin, Piperine, Usnic acid, Lecanoric acid, Cedrelanol, Umbelliferone, Myristicin, Alpha-terpinyl acetate, and Coumarin) were chosen for the molecular docking studies with AutoDock tools 1.5.6.

Table 6: Binding energies of ligands

Serial No.	Phytochemicals	PUBCHEM ID	Binding energy after docking with 5KIR (kcal/mol)
1.	Farnesiferol A	7067262	–9.8
2.	Atranorin	68066	–9.4
3.	Piperine	638024	–8.6
4.	Usnic acid	5646	–8.5
5.	Lecanoric acid	99613	–8.5
6.	Cedrelanol	160799	–8.3
7.	Umbelliferone	5281426	–7.7
8.	Alpha-terpinyl acetate	111037	–7.4
9.	Coumarin	323	–7.4
10.	Myristicin	4276	–7.0
11.	Ferulic acid	445858	–6.9
12.	Beta caryophyllene	5281515	–6.8
13.	Capsaicin	1548943	–6.8
14.	Limonene	22311	–6.7
15.	Terpinolene	11463	–6.7
16.	Eugenol	3314	–6.7
17.	Cuminaldehyde	326	–6.5
18.	Trans anethole oxide	10080713	–6.5
19.	Estragole	8815	–6.4
20.	Fenchone	14525	–6.3
21.	Alpha-phellandrene	7460	–6.3
22.	Fenchone	14525	–6.3
23.	Cinnamaldehyde	637511	–6.1
24.	Linalyl acetate	8294	–6.0
25.	Thymoquinone	10281	–6.0
26.	Elemicin	10248	–6.0
27.	Safrole	5144	–5.9
28.	Anisaldehyde	31244	–5.7
29.	Eucalyptol	2758	–5.6
30.	Linalool	6549	–5.5
31.	Alpha-pinene	6654	–5.2
32.	Myrcene	31253	–4.8
Known inhibitors			
33.	Celecoxib	2662	–12.0
34.	Rofecoxib	5090	–7.7

Brain or intestinal estimated permeation method (BOILED EGG)

From the analysis, Lecanoric acid, Atranorin, Usnic acid, and Celecoxib are predicted to be well absorbed but not accessing the brain and are PGP, whereas Piperine, Alpha terpinyl acetate, Coumarin, Cedrelanol, Myristicin, Umbelliferone, and Farnesiferol A are predicted to be brain-penetrant. Farnesiferol A is the only ligand capable of getting pumped out through P glycoprotein transporters and the rest of them are not subject to active efflux.

Docking results

Molecular docking studies of COX-2 protein were performed using AutoDock tools 1.5.6 with these 12 ligands (10 selected phytocontituents, Celecoxib, and Rofecoxib) using Lamarckian genetic algorithm. Among the 10 runs, the most favorable configurations were selected from cluster RMSD table for further analysis. Docking result is presented in Table 7.

Docking results reveal that binding energies of Celecoxib and Rofecoxib (prescribed drugs against COX-2) with COX-2 enzyme are –7.55 and –7.96 kcal/mol, respectively. Lower the binding energy more stable is the binding. On the basis of binding energies, 4 compounds are showing binding energies either close to or lower than the "coxib" drugs and hence could be promising drug candidate against COX-2 target. These 4 compounds are Farnesiferol A, Piperine, Usnic acid, and Cedrelanol with binding energies –9.39, –7.58, and –7.51, –7.40, respectively. Structure of all the 4 compounds along with the coxib drugs is shown in Fig. 3.

Farnesiferol A

Farnesiferol A, an active constituent of asafoetida of spice-mix, showed best binding with COX-2 enzyme. It can form various kinds of bonds with COX-2 protein ranging from conventional hydrogen bonds, pi-donor hydrogen bonds, and a range of hydrophobic interactions with Pro, Phe, Leu, and Gln side chains. Some important hydrophobic interactions and hydrogen bonds are listed in Table 8, obtained from PLIP.

Piperine

Piperine is an active constituent of pepper, showed second best binding. It forms conventional hydrogen bonds with Arg, Val residue (with oxygen atom in ligand), pi-alkyl bond with Phe142 and a range of hydrophobic interactions with Phe (142A,142B), Gln (374A,374B), and Val538 side chains. Some important hydrophobic interactions and hydrogen bonds are listed in Table 9.

Usnic acid

Usnic acid, active constituent of stone flower, showed third best binding. It forms conventional hydrogen bonds with COX-2 protein, along with pi-alkyl hydrophobic interactions with Trp, Phe, His, and Gln side chains. Oxygen atoms in ligand molecule are involved in conventional hydrogen bonds with Arg-376A, Asn-375, Leu-224, and Gly-225. Some important hydrophobic interactions and hydrogen bonds are listed in Table 10.

Table 7: Molecular docking results

Sl. No.	Protein	Ligands	Cluster RMSD	Reference RMSD	Binding energy (–kcal/mol)	Inhibitory constant (Ki)
1.	COX-2 (5KIR)	Farnesiferol A		44.96	–9.39	131.48nM
2.		Piperine		43.40	–7.58	2.76 μM
3.		Usnic acid		45.10	–7.51	3.15 μM
4.		Cedrelanol		33.23	–7.40	3.79 μM
5.		Lecanoric acid		41.43	–7.29	4.54 μM
6.		Atranorin		45.73	–6.35	22.17μM
7.		Umbelliferone		35.23	–6.01	39.40 μM
8.		Coumarin		32.20	–5.74	62.14 μM
9.		Myristcin		43.09	–5.71	65.11 μM
10.		Alpha terpinyl acetate		42.39	–5.40	110.02μM
11.		Rofecoxib		45.78	–7.96	1.47 μM
12.		Celecoxib		46.03	–7.55	2.94 μM

Table 8: Receptor-ligand interaction of Farnesiferol A with COX-2 protein

Hydrophobic Interactions					
Index	**Residue**	**Arachidonic acid**	**Distance**	**Ligand atom**	**Protein atom**
1	142A	PHE	3.66	10864	1088
2	142B	PHE	3.44	10868	6515
3	145A	LEU	3.88	10860	1118
4	145B	LEU	3.63	10863	6542
5	374A	GLN	3.91	10868	3398
6	374B	GLN	3.74	10855	8816

Hydrogen Bonds							
Index	**Residue**	**Arachidonic acid**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Donor Atom**	**Acceptor Atom**
1	375B	ASN	1.80	2.58	134.20	10878 [O3]	8827 [O2]
2	376B	ARG	2.01	2.80	151.37	8842 [Ng+]	10878 [O3]
3	376B	ARG	3.39	3.92	121.91	8845 [Ng+]	10878 [O3]
4	538A	VAL	1.74	2.60	175.15	5019 [Nam]	10873 [O2]

Table 9: Receptor-ligand interaction of piperine with COX-2 protein

Hydrophobic Interactions					
Index	**Residue**	**Arachidonic acid**	**Distance**	**Ligand Atom**	**Protein Atom**
1	142A	PHE	3.08	10871	1090
2	142A	PHE	3.49	10851	1088
3	142B	PHE	3.87	10861	6515
4	374A	GLN	3.42	10862	3398
5	374B	GLN	3.82	10863	8816
6	538B	VAL	3.88	10870	10441

Hydrogen Bonds							
Index	**Residue**	**Arachidonic acid**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Donor Atom**	**Acceptor Atom**
1	375B	ASN	2.62	3.19	124.95	8824 [Nam]	10866 [O3]
2	376A	ARG	2.18	3.02	166.70	3424 [Ng+]	10856 [O2]
3	376A	ARG	3.26	3.87	130.82	3427 [Ng+]	10856 [O2]
4	538B	VAL	3.04	3.40	107.96	10437 [Nam]	10869 [O3

Table 10: Receptor-ligand interaction of piperine with COX-2 protein

Hydrophobic Interactions					
Index	**Residue**	**Arachidonic acid**	**Distance**	**Ligand Atom**	**Protein Atom**
1	139B	TRP	3.85	10867	6484
2	142B	PHE	3.87	10875	6515
3	142B	PHE	3.57	10867	6514
4	226A	HIS	3.59	10860	1895
5	374A	GLN	3.64	10862	3398

Hydrogen Bonds							
Index	**Residue**	**Arachidonic acid**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Donor Atom**	**Acceptor Atom**
1	224A	LEU	2.13	2.97	143.57	10871 [O3]	1880 [O2]
2	225A	GLY	3.36	3.82	111.15	10876 [O3]	1889 [O2]
3	375A	ASN	2.00	2.86	170.96	3406 [Nam]	10851 [O3]
4	376A	ARG	3.37	3.97	129.11	3424 [Ng+]	10853 [O2]
5	376A	ARG	2.15	2.97	160.54	3427 [Ng+]	10853 [O2]

Cedrelanol

Cedrelanol is an active constituent of cinnamon of spice-mix. It forms conventional hydrogen bonds with Gln-372 and Lys-532 residues, pi-alkyl hydrophobic interactions with Thr-118, Gln-370, Phe- 371, and Lys- 532 side chains. Some important hydrophobic interactions and hydrogen bonds are listed in Table 11.

Celecoxib

Celecoxib is a non-steroidal anti-inflammatory drug used as COX-2 inhibitor. It is used here as a control for screening potential drug molecules from the spice-mix. Docking results reveal that it can form various kinds of bonds ranging from conventional hydrogen bonds involving oxygen and nitrogen atom with Asn-375, Arg-376, and Gln-374 residues; hydrophobic interactions with Phe-142, Leu-145, and Gln-374 residue; pi stacking interaction with Phe-142A; and halogen bond involving Fluorine with His-226 residue. Some important hydrophobic interactions and hydrogen bonds are listed in Table 12.

Rofecoxib

Rofecoxib is also a nonsteroidal anti-inflammatory drug used as COX-2 inhibitor. It is also used as a control for screening potential drug

Table 11: Receptor-ligand interaction of cedrelanol with COX-2 protein

Hydrophobic Interactions					
Index	**Residue**	**Arachidonic acid**	**Distance**	**Ligand Atom**	**Protein Atom**
1	118A	THR	3.98	10862	847
2	370A	GLN	3.70	10865	3348
3	371A	PHE	3.32	10857	3362
4	372A	GLN	3.29	10855	3373
5	532A	LYS	3.50	10860	4973

Hydrogen Bonds							
Index	**Residue**	**Arachidonic acid**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Donor Atom**	**Acceptor Atom**
1	372A	GLN	2.07	2.91	166.54	3368 [Nam]	10866 [O3]
2	532A	LYS	1.89	2.73	154.71	4975 [N3+]	10866 [O3]

Table 12: Receptor-ligand interaction of Celecoxib with COX-2 protein

Hydrophobic Interactions					
Index	**Residue**	**Arachidonic acid**	**Distance**	**Ligand Atom**	**Protein Atom**
1	142A	PHE	3.05	10868	1090
2	142A	PHE	3.78	10867	1088
3	142A	PHE	3.65	10857	1089
4	145A	LEU	3.37	10862	1117
5	145B	LEU	3.36	10861	6542
6	374B	GLN	3.90	10864	8816

Hydrogen Bonds							
Index	**Residue**	**Arachidonic acid**	**Distance H-A**	**Distance D-A**	**Donor Angle**	**Donor Atom**	**Acceptor Atom**
1	374A	GLN	2.07	2.80	126.13	10872 [N3]	3401 [O2]
2	375A	ASN	2.41	3.13	140.92	3406 [Nam]	10870 [O2]
3	375B	ASN	3.19	3.88	138.35	8824 [Nam]	10852 [Nar]
4	376A	ARG	2.15	3.01	173.49	3424 [Ng+]	10871 [O2]
5	376A	ARG	3.13	3.77	133.03	3427 [Ng+]	10871 [O2]

π-Stacking							
Index	**Residue**	**Arachidonic acid**	**Distance**	**Angle**	**Offset**	**Stacking Type**	**Ligand Atoms**
1	142A	PHE	5.13	68.75	1.72	T	10851, 10852, 10853, 10855, 10856

Halogen Bonds							
Index	**Residue**	**Arachidonic acid**	**Distance**	**Donor Angle**	**Acceptor Angle**	**Donor Atom**	**Acceptor Atom**
1	226B	HIS	3.02	143.77	145.31	10876 [F]	7312 [O2]

Table 13: Receptor-ligand interaction of Rofecoxib with COX-2 protein

Hydrophobic Interactions					
Index	**Residue**	**Arachidonic acid**	**Distance**	**Ligand Atom**	**Protein Atom**
1	142A	PHE	3.83	10859	1090
2	145A	LEU	3.37	10869	1118
3	145A	LEU	3.73	10870	1117
4	145B	LEU	3.61	10872	6542
5	226B	HIS	3.81	10862	7313
6	374B	GLN	3.61	10858	8816

Salt Bridges						
Index	**Residue**	**Arachidonic acid**	**Distance**	**Protein positive?**	**Ligand Group**	**Ligand Atoms**
1	376B	ARG	3.70	yes	Carboxylate	10851, 10852

molecules from the spice-mix. Docking results reveal that it also can form various kinds of bonds ranging from a salt bridge formation with Arg- 376 residue; a range of hydrophobic interactions such as pi-sigma (Leu-145B), pi-sulphur (Trp 139A), and pi-alkyl bond (Leu-145A) as shown in Table 13.

CONCLUSION

This study reveals that "Garam masala;" the routinely used spice-mix in Indian cuisine; harbor certain active constituents with anti-inflammatory potential against COX-2 target. Docking result ascertains

that Farnesiferol A shows lower binding energy than both of the commercially used drug (Celecoxib and Rofecoxib) and the rest three (Piperine, Cedrelanol, and Usnic acid) molecules bind with COX-2 with almost similar binding energies like those of "coxibs." This makes them potential drug candidates against COX-2 target. Along with that, drug likeness analysis using *Lipinski's filter* (*Rule of Five*) of the compounds allowed them for human consumption. ADMESAR analysis of pharmacokinetic properties and BOILED-Egg analysis further strengthen our findings. Commercially used "coxibs" impart certain side effects to our body which makes it all the more important to utilize the properties of natural compounds and explore the possibility of using them as potential drugs. Even though, the binding stability of the natural compounds might need to be improved before using them as potential drugs, this preliminary assessment might lead to the future research of designing rational derivatives from the natural compounds found in spices, which can eventually substitute the commercially available drugs that cause side effects.

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AUTHOR CONTRIBUTIONS

S.D reviewed different literatures and put forward the idea. V.B. and S.D. designed the framework of docking experiment. S.D. performed the experiments, analyzed data, and wrote the paper. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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