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Evaluation of Selected Natural Compounds for Cancer Stem Cells Targeted Anti-cancer Activity: A Molecular Docking Study

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Authors' contributions

This work was carried out in collaboration between all authors. Author SRS designed the overall research. Author KM carried out all the experiments including, docking, scoring, ranking and analysis, and drafted the manuscript. Authors SRS, KHT and AS provided and suggested natural ligands list and supervised the study. Author JRV performed the DOCK6 calculations and classification of the docked poses. Authors SRS, KHT and JRV corrected the manuscript. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Aims: Cancer stem cells (CSCs) play significant roles in tumor initiation, relapse, angiogenesis, metastasis and therapy. Collectively Wnt, Notch, and Hedgehog are major pathways that have been linked to the drug resistance of CSCs. Eliminating CSCs has been suggested as a promising approach to cure cancer. Aim of the present study is screening of selected natural compounds for inhibitors of Wnt, and Hedgehog pathways that have been linked to the drug resistance of cancer stem cells (CSCs) by *in silico* molecular docking analysis.
Place and Duration of Study: At the Institute of Biochemistry, Molecular Biology and

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Biotechnology, University of Colombo between 1st of June 2014 to May 2015.
Methodology: In the present study, *in silico* molecular docking simulations were carried out for the binding of 35 selected natural compounds with receptor proteins which are involved in the main signaling pathways of CSCs, such as β-catenin chain A and Smo receptor from the Wnt and hedgehog pathways respectively, using Hex 8.0.0, DOCK6 and AutoDock Vina software. Docking interaction residue analysis, score functions and drug-likeness studies were carried out for the selected compounds.
Results: Overall, 11 compounds such as Gedunin, Kaempferol, Methylripariochromene A, Myrigalone G, Catechin, Myricetin, Discretine, Laurolitsine, Myricitrin, Nordicentrine and Phloretin were identified with good binding energy, interaction, binding affinity and better drug likeness for β-catenin chain A. There was no considerable overall binding ability for Smo inhibition.
Conclusion: Our results demonstrate that 11 compounds out of 35 natural compounds screened can be used for further development of CSC targeted anti-cancer drugs. *In vitro* studies need to be carried out to confirm anti-CSC activity of the novel inhibitors discovered.

Keywords: Cancer stem cells; molecular docking; gedunin; Wnt; Hedgehog.

1. INTRODUCTION

The CSCs hypothesis proposes that a small subset of cells is responsible for the initiation, extensive proliferation and metastasis of a tumor [1,2]. CSCs have been identified in a variety of tumors including breast, skin, blood, brain, colon, pancreatic, prostate, ovarian, liver and lung cancers [3]. Furthermore, these cells are highly resistant to conventional chemotherapy [4]. Most therapies are being targeted to fast growing tumor mass. However, they are not targeted to the slow dividing CSCs. If CSCs remain following cancer therapy they can lead to recurrence of the cancer. Use of therapies with targeted CSC-specific agents may target the whole cancer and minimizes long-term effects such as recurrence and metastasis. Dysregulation of signaling pathways such as Wnt and Hedgehog networks plays an important role in enabling CSCs to retain stem cell properties.

The Hedgehog (Hh) signaling pathway is activated when Shh ligand binds to the transmembrane patched proteins. In the absence of the ligand, PTCH1 (Patched receptor) represses Smo (Smoothened), which prevents the activation of Hedgehog. However, after binding of Shh to PTCH1, the interaction of PTCH1 and Smo receptor is altered and Smo receptor is inhibited. Activated Smo receptor activates several members of the GLI (Glioma-associated oncogene homologue) family of transcription factors into active forms. Subsequently, active form of GLI family of transcription factors translocate into the nucleus activating the GLI-targeted genes that control the embryonic development and self-renewal nature of CSCs [5,6]. The Hh signaling pathways proteins, PTCH1 and Smo receptors may provide new therapeutic options for many different cancer types [7] and a small-molecule Smo inhibitor had been used in clinical oncology [8].

Wnt signaling pathway plays a key role in tumor cell de-differentiation and proliferation [9]. Wnt/β-catenin pathway is divided into two main categories based on their role in cytosolic β-catenin stabilization and activation of specific receptors: these are canonical and non-canonical Wnt signaling [10]. Aberrant canonical Wnt signaling activation, such as those following mutations in components of the Wnt/β-catenin are often associated with cancer [11]. Aberrant activation of Wnt signaling leads to β-catenin translocation to the nucleus resulting in expression of target genes where it binds to T cell factors/lymphoid enhancer factor (TCF/LEF) transcription factor family. If the process is uncontrolled due to inactivation of destruction complex, there will be a continuous supply of nonphosphorylated β-catenin to the nucleus leading to over-expression of genes. β-catenin drives transcription of programs critical for CSCs and tumor cells. β-catenin, as a co-activator, in complex with trans acting TCFs or LEF-1 is a cause of a wide variety of carcinomas. Inhibition of this complex may lead to prevention of transcriptional activation of β-catenin/TCF target genes, thereby serving as a therapeutic agent. Uncontrolled transcription in cancer cells caused by the activity of β-catenin can be avoided by prevention of β-catenin /TCF complex formation [12].

Numerous types of bioactive compounds, widely used in cancer chemotherapy have been isolated from plant sources. Development of preclinical

research *in vitro* always needs to be followed up by *in vivo* experiments, toxicological experiments, and clinical trials and the total development process of a new anticancer drug can take a very long time. A rational modern approach in drug discovery research is to use *in silico* virtual screening methods for the development of new drug leads [13]. Structure-based drug design uses an efficient and intelligent approach to design improved ligands for the target [14]. The robustness of any computational approach depends on how accurately the experimental information is derived and parameterized to simulate a biological system.

In the present study, Molecular docking analysis was used to identify natural drug leads that can efficiently target CSCs signalling pathways' receptor proteins, such as β-catenin chain A and the Smo receptor from the Wnt and hedgehog pathways respectively.

2. MATERIALS AND METHODS

2.1 Protein Preparation

The β-catenin (PDB ID: 1JDH) and Smo receptor (PDB ID: 4JKV) were identified as the target protein of Wnt and Hedgehog signalling pathways respectively and these retrieved from PDB. The selected proteins were prepared for docking by selecting and deleting the water molecules and all unnecessary ligands which are already existing in the selected proteins using the script option of Discovery Studio 4.0 Visualizer software (Accelyrs Software Inc., Discovery Studio Modeling Environment, and Release 4.0.San Diego: Accelrys Software Inc., 2013). In β-catenin protein (1JDH), chain A was selected for the docking analysis with selected natural compounds and chain B was removed using UCSF Chimera 1.8.1 [15]. The same procedure was carried out to remove the already existing antitumor agent (LY2940680) in the human Smo receptor. The DOCKPrep protocol was followed by inserting missing atoms in incomplete residues, deleting alternate conformations, removing solvents, removing incomplete side chains, adding charges and adding hydrogen. All these operations were carried out using UCSF Chimera 1.8.1. The modified proteins were saved in MOL2 format for AutoDock Vina [16] and in PDB format for Hex dock respectively.

2.2 Ligand Preparation

β-catenin reference ligand of hTCF-4 (human T cell factor) Chain B and Smo receptor reference ligand of antitumor agent LY2940680 were extracted from 1JDH and 4JKV respectively. Natural ligands to be studied were obtained by a literature survey with priority being given to the compounds isolated from plants grown in Sri Lanka or Asian region. 3D structures of some natural compounds were downloaded from ZINC data base [17]. Canonical SMILES notations of chemical structures of some of the other selected compounds were collected from the PubChem [18] and ChemSpider databases [19] and were drawn using the ACD/ChemSketch software. Finally, the collection of structure formats were converted to specific required format, namely PDB for the Hex docking software and MOL2 format for DOCK6, AutoDock Vina using Accelrys discovery studio 4.

2.3 Ligand Optimization

Optimization of the selected 35 natural ligands was carried out by defining atom types using their connectivity, optimizing the chemical structure using a molecular dynamics conformational search followed by molecular mechanics optimization of the 10 best conformations and selection of the minimal energy result using by Gabedit 2.4.8 [20]. It was further refined by a final optimization using openMOPAC [21]. Finally best conformation generated was saved in PDB or MOL2 for the docking studies.

2.4 Prediction of Protein Binding Pocket

The binding pockets of β-catenin chain A and the human Smo receptor were predicted using the CASTp server [22].

2.5 Docking by Hex 8.0.0

Initially docking of the reference ligand hTCF-4 with active site of the β-catenin chain A and the reference ligand, anti-tumor agent LY2940680 against the active site of the human Smo receptor were carried out using Hex 8.0.0 [23] to get a validity check. Subsequently natural compounds were docked against β-catenin chain A and human Smo receptor active sites.

2.6 UCSF DOCK6 Docking and Scoring

All selected natural molecules were docked against the two selected target proteins (β-catenin chain A and Smo receptor) with DOCK6 [24] on the CNB/CSIC computing cluster. Rigid and flexible dockings were carried out in both cases. Pose generation was followed by a subsequent ligand optimization step. Hence, the final results from rigid docking also allow for ligand flexibility, although the pose search is not as powerful as in the flexible docking approach. Generated poses were then scored using DOCK6 Grid score, a force-field based score using non-bonded ligand-receptor interaction terms. Among them, the best scoring pose from flexible docking was subjected to molecular mechanics minimization in the active site and scoring using Hawkins' GB/SA [25] a function that considered the interaction energies and the energetic cost required to modify the shape of the ligand and the cost of displacing water from the active site. Of course, if flexible docking fails, this Hawkins' GB/SA function also fails. Results were further re-scored using X-score [26] and Drug Score [27] and visualized using the UCSF Chimera ViewDock tool to inspect the poses, calculate contacts and H bonds and select the best scoring conformers. Finally, PCS (Per Center Score) and PCS known/PCS ligand were calculated for affinity checking.

2.7 Docking Analysis by AutoDock Vina

The most promising compounds were also subject to docking analysis against the target proteins using AutoDock Vina in combination with UCSF Chimera 1.8.1.

2.8 Docking Interaction Analysis

The strong binding site residues of β-catenin chain A with human TCF transcription factor (reference ligand) and residues of Smo receptor with anti-tumor agent LY2940680 were computationally predicted using Accelrys Discovery studio 4.0. With this knowledge, an analysis of binding site residues for β-catenin chain A and Smo receptor with selected natural compounds was performed from the Hex docked complex. Interactions were expected upon binding with each proteins active site.

2.9 Drug Likeness Analysis

Various basic physico chemical properties of drug likeness of the natural compounds were retrieved from the ZINC, PubChem, and ChemSpider databases and the rest were computed using MarvinSketch - ChemAxon software.

3. RESULTS AND DISCUSSION

3.1 Protein Preparation

Fig. 1 represents structural views of receptors and their reference ligands (hTCF-4 bound to β-catenin and the human Smo receptor 7TM domain in complex with LY2940680).

3.2 Prediction of Protein Binding Pocket

Binding pockets of β-catenin chain A and the human Smo receptor predicted from the CASTp server are shown in Fig. 2.

3.3 Ligand Preparation

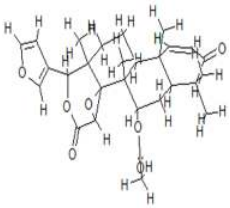
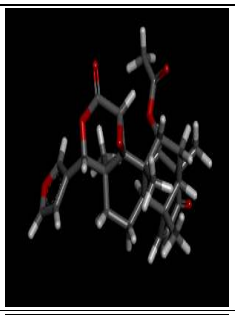
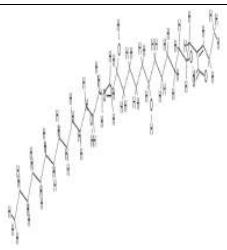
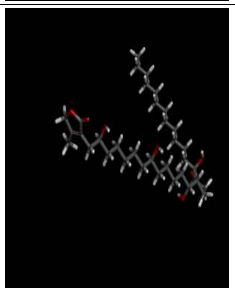
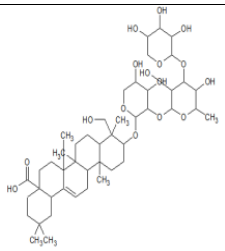
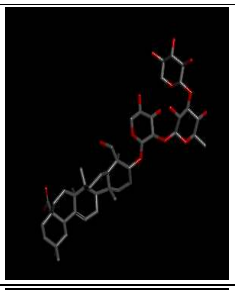
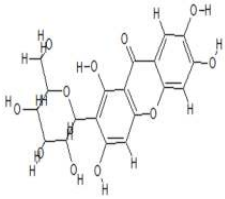
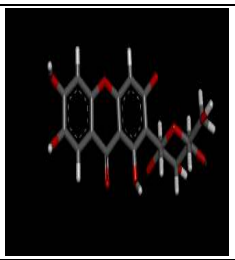
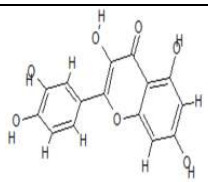
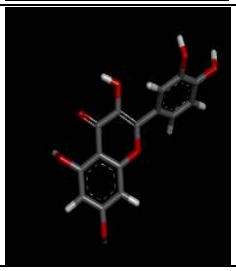
Table 1 depicts 35 natural ligands isolated from medicinal plants grown in Sri Lanka or Asian countries.

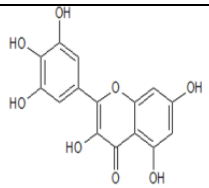
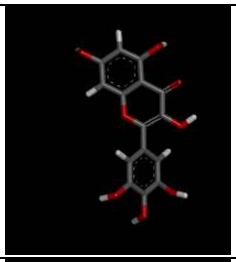
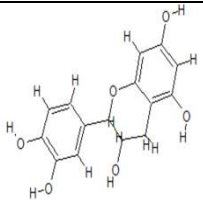
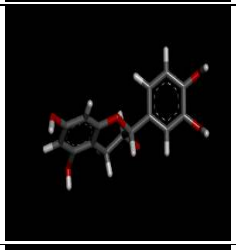
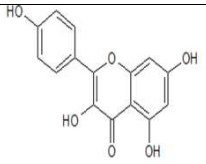
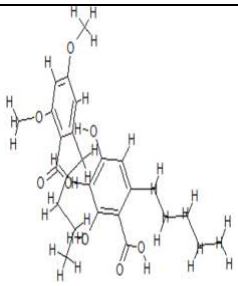
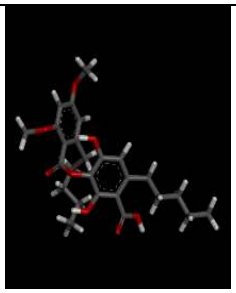
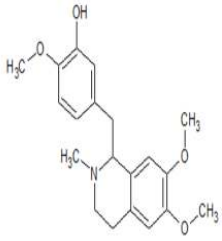
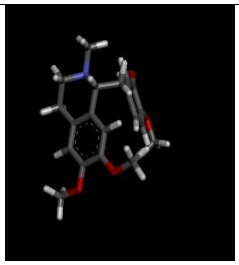
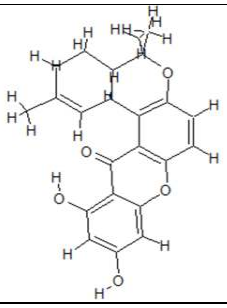
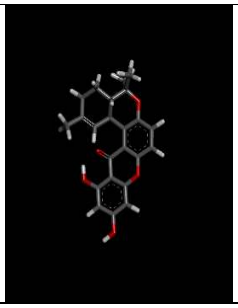
3.4 Molecular Docking Analysis

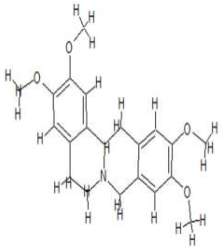
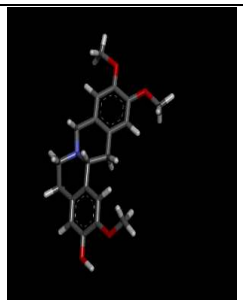
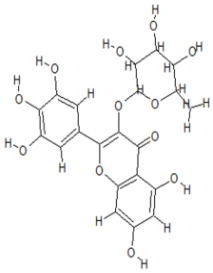
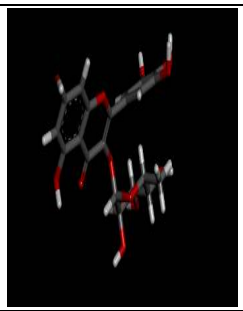
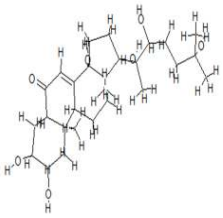
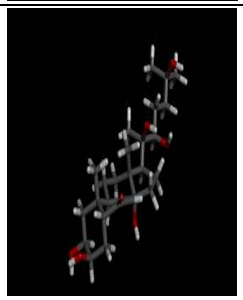
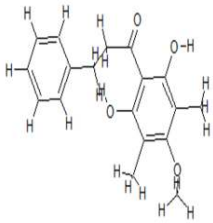
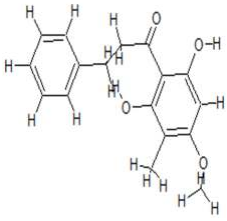
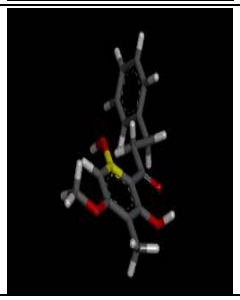
The molecular docking analysis was initially carried out using the Hex 8.0.0 and DOCK6 docking software for selected natural compounds (Table 1). Based on the preliminary results of Hex 8.0.0 and DOCK6 analysis, docking interaction analysis and drug likeness study of selected compounds (11 compounds) were subjected for further docking analysis by using AutoDock Vina docking software. The whole docking procedure was carried out, following a series of sequential steps. Initially hTCF-4 (chain B) was docked against β-catenin (chain A) and the anti-tumor agent LY2940680 was docked against the Smo receptor using the Hex docking software, to obtain the reference docking energy of the complex of β-catenin with hTCF-4. The reference docking energy value was found to be -818.23 and of the complex of Smo receptor docked with its anti-tumor agent, was found to be -338.62 as summarized in Table 2. Thereafter, the natural compounds (35 compounds) were docked with β-catenin chain A and the Smo receptor separately. The resultant Hex docking energy values for the natural compounds are summarized in Table 3. The docking scores obtained with DOCK6 are summarized in Table 4 and Table 5. Docking scores are loosely related to binding energies, with lower scores suggesting more stable receptor-ligand complexes and

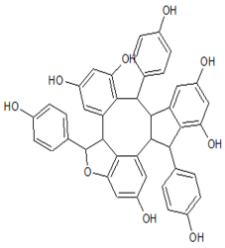
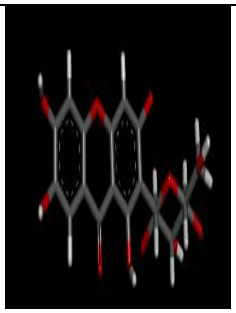
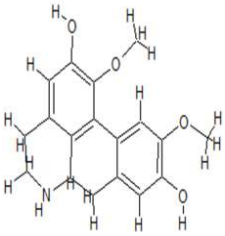
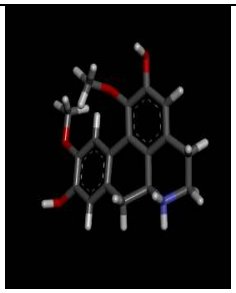
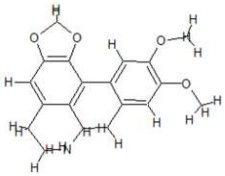
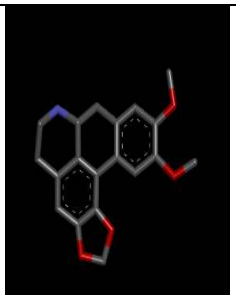
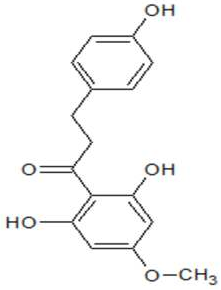
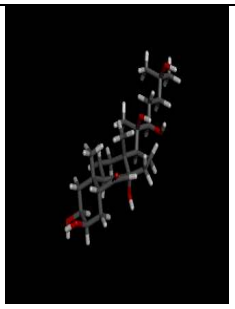
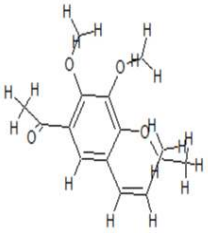
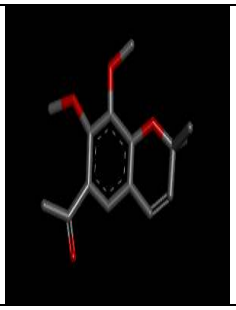
greater binding affinity of each natural product for a given receptor. Therefore low energy depicts a more stable conformation because a greater energy input would be needed to dissociate the complex resulting in a stable binding of the ligand to the receptor [46].

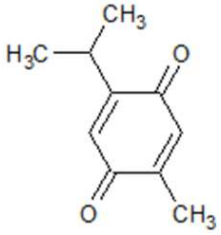
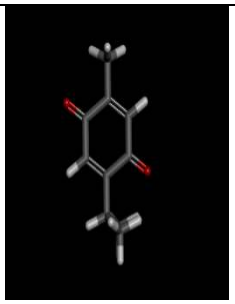
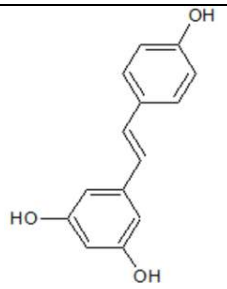
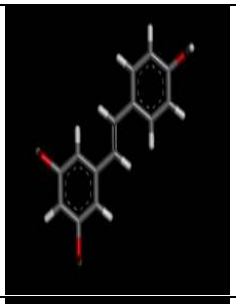
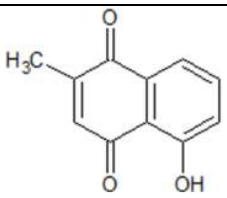
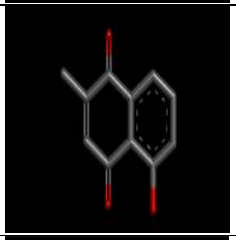
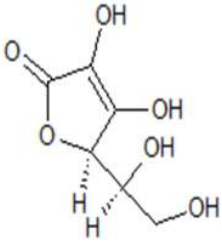
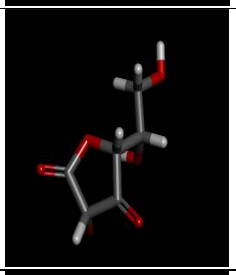
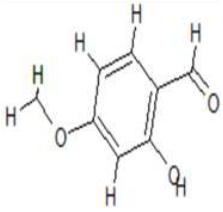
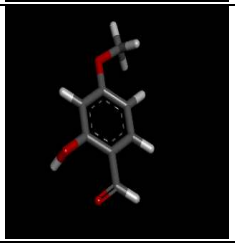
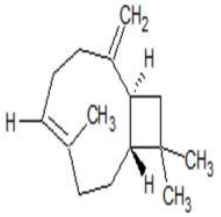
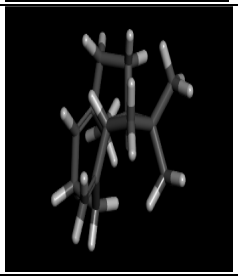
Table 1. Chemical structures of selected natural compounds used for the docking analysis

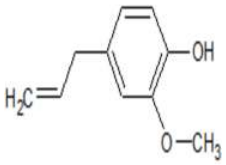
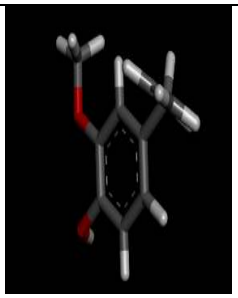
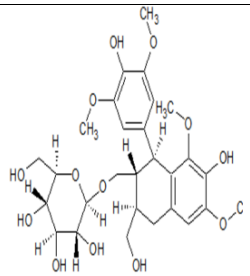
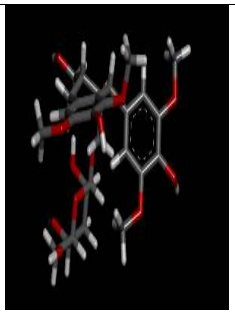
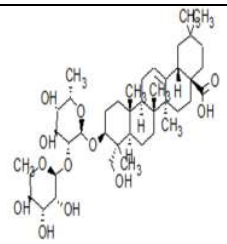
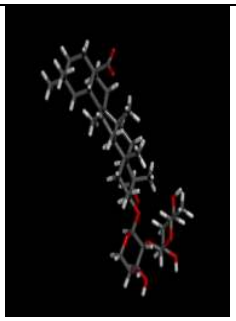
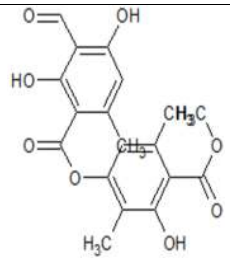
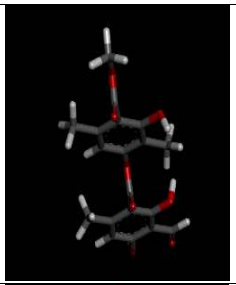
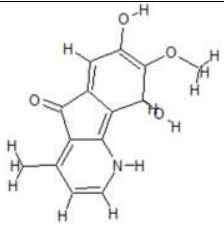
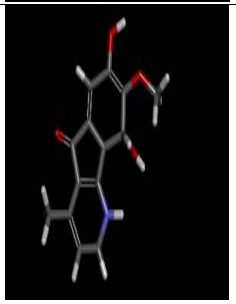
Name of the ligand	2D Structure	3D Structure	Plant source	References
Gedunin			*Azadirachta Indica*	[28]
Annonacin			*Annona muricata*	[29]
Kalopanax saponin I			*Nigella sativa*	[30]
Mangiferin			*Mangifera indica*	[31]
Quercetin			*Osbeckia aspera*	[32]

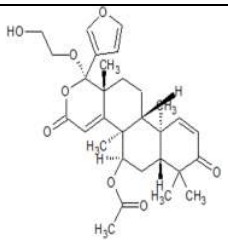
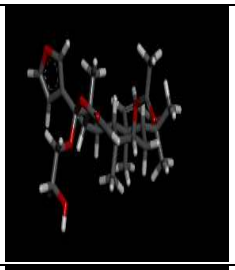
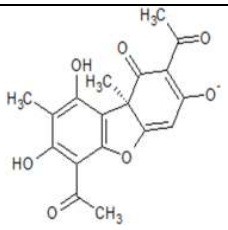
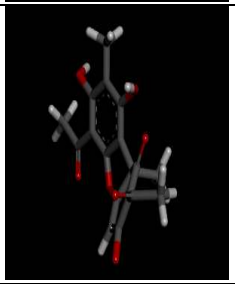
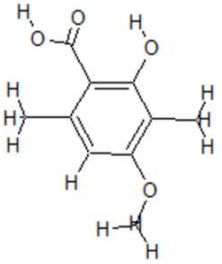
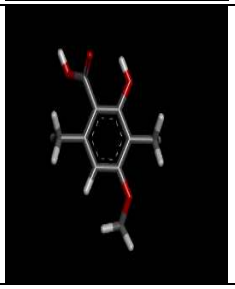
Name of the ligand	2D Structure	3D Structure	Plant source	References
Myricetin			*Camellia sinensis*	[33]
Catechin			*Camellia sinensis*	[33]
Kaempferol			*Osbeckia aspera*	[33]
4-O-methyl cryptochlorophaeic acid			*Pyxine consocians*	[34]
Laudanidine			*Xylopia championii*	[34]
Calozeyloxanthone			*Calophyllum moonii*	[34]

Name of the ligand	2D Structure	3D Structure	Plant source	References
Discretine			*Xylopia championii*	[35]
Myricitrin			*Elaeocarpus serratus* L	[34]
Ecdysterone			*Achyranthes aspera*	[36]
Myrigalone B			*Syzygium jambos* L	[34]
Myrigalone G			*Syzygium jambos* L	[34]

Name of the ligand	2D Structure	3D Structure	Plant source	References
Canaliculatol			*Stemonoporous canaliculatus*	[34]
Laurolitsine			*Litsea gardneri* L	[34]
Nordicentrine			*Xylopia championii*	[35]
Phloretin			*Syzygium jambos* L	[35]
Methylripariochromene A			*Eupatorium riparium* Regel	[34]

Name of the ligand	2D Structure	3D Structure	Plant source	References
Thimoquinone			*Nigella sativa*	[2]
Resveratrol			*Vitis vinifera*	[37]
Plumbagin			*Plumbago zeylanica*	[38]
Ascorbic acid			*Solanum lycopersicum*	[39]
2-Hydroxy-p-anisaldehyde			*Hemidesmus indicus*	[40]
β-Caryophyllene			*Cannabis sativa* L	[41]

Name of the ligand	2D Structure	3D Structure	Plant source	References
Eugenol			*Cinnamomum verum*	[42]
(+)-lyoniresinol-3-a-O-ß-D glucopyranoside			Lycium barbarum L	[43]
1-Alpha hederin			*Nigella sativa*	[30]
Atranorin			*Heterodermia microphylla*	[34]
Cyathocaline			*Cyathocalyx zeylanica*	[34]

Name of the ligand	2D Structure	3D Structure	Plant source	References
Mahmoodin			*Azadirachta Indica*	[44]
Usnic acid			*Usnea sp*	[34]
3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid			*Leproloma sipmantanum*	[45]

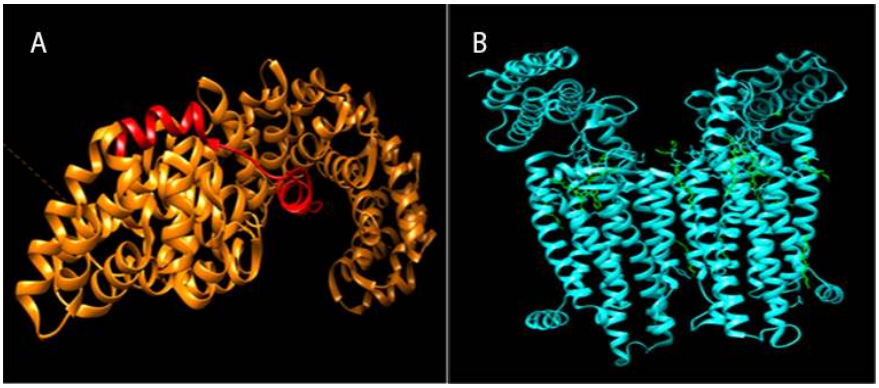


Fig. 1. Overall structure of hTCF-4 bound to β-catenin and the human Smo receptor 7TM domain in complex with LY2940680

(A) Interactions between hTCF-4 (red) and β-catenin(orange), (B) The Smo receptor which has crystallized as a dimer in an asymmetric unit (cyan), LY2940680 and lipids (forest green)

Table 2. Docking analysis of β-catenin chain A and Smo receptor with their original reference ligands resulted from Hex 8.0.0

Original reference docked complex	E-value
β-catenin chain A with TCF-4 chain B	-818.23
Smo receptor with anti-tumor agent	-338.62

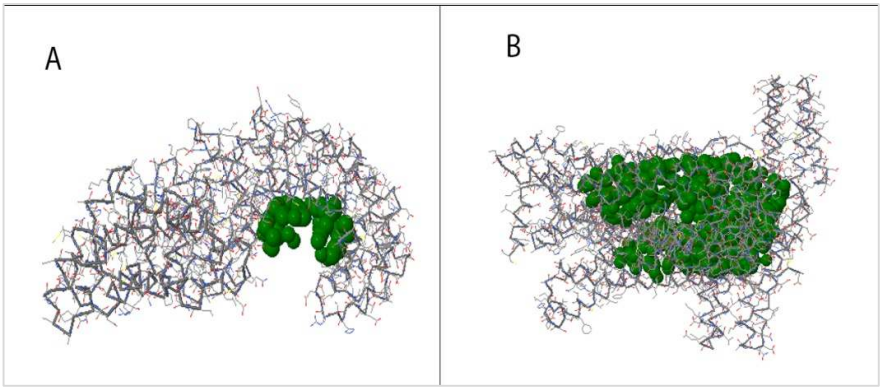


Fig. 2. Binding pockets of β-catenin chain A and the human Smo receptor
(A) Identified binding pocket structure of beta-catenin chain A which has bound with human TCF-4 protein. (B) Identified binding pocket structure of the human Smo in complex with antitumor agent LY2940680

Table 3. Docking analysis of selected natural compounds with β-catenin chain A and Smo receptor resulted from Hex 8.0.0

Ligands	Docking E-Value with β-catenin chain A	Docking E-Value with Smo receptor
Kalopanax saponin I	-368.92	-404.52
Canaliculatol	-319.09	-348.85
1-Alpha hederin	-298.06	-348.80
(+)-lyoniresinol-3-a-O-ß-D-glucopyranoside	-290.16	-315.33
Myricitrin	-278.26	-301.79
Methylripariochromene A	-277.87	-258.15
Mangiferin	-267.51	-328.71
Myricetin	-257.23	-271.79
Discretine	-253.36	-273.88
Gedunin	-249.73	-275.30
Mahmoodin	-250.12	-298.68
Nordicentrine	-248.86	-269.30
Catechin	-248.43	-247.07
Myrigalone G	-246.10	-248.44
Laudanidine	-244.36	-262.49
Ecdysterone	-241.42	-307.26
Kaempferol	-236.11	-244.93
Laurolistine	-231.83	-255.35
Phloretin	-212.98	-238.10
β-Caryophyllene	-164.15	-195.01
Ascorbic acid	-163.73	-184.99
Annonacin	-348.98	-410.04
Quercetin	-252.23	-254.65
4-o-methyl-cyptochorophaeic acid	-261.44	-314.86
Calozeyloxanthone	-235.28	-281.97
Myrigalone B	-61.52	-51.13
Thimoquinone	-153.84	-201.07
Resveratrol	-209.16	-228.35
Plumbagin	-125.88	-81.90
2-Hydroxy-p-anisaldehyde	-157.56	-183.93
Eugenol	-172.79	-188.32
Atranorin	-256.60	-286.93
Cyathocaline	-177.87	-140.33
Ursonic acid	-233.57	-240.96
3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid	-181.67	-195.79

Table 4. DOCK6 docking analysis of selected natural compounds with the β- catenin receptor chain A (Grid score, Drug Score, X score, PCS, RMSD and PCS ligand/PCS reference ligand)

Ligands	Grid score	Hawkins GB	X-score	Drug Score	PCS	RMSD	PCS ligand/PCS reference
Ref- β catenin with TCF-4			-9.59	-243.543	-0.117	0	
(+)-lyoniresinol-3-a-O-ß-D-glucopyranoside- Rigid	-50.7685		-6.57	-55.615	-0.114	10.34	0.97
1-alpha-hedarin-Rigid	-59.6167		-8.48	-84.046	-0.152	16.51	1.30
2-Hydroxy-p-anisaldehyde-Rigid	-9.2456		-5.64	-31.886	-0.055	4.407	0.47
3,6-dimethyl-2-hydroxy-4-methoxybenzoic_acid - Rigid	-36.3504		-6.47	-42.336	-0.157	55.96	1.34
4-o-methyl-cyptochorophaeic_acid - Rigid	-40.6463		-6.2	-57.29	-0.119	22.73	1.02
Annonacin- Rigid	-45.3335		-5.21	-71.135	-0.18	7.412	1.54
Atranorin- Flex	-45.2722	-17.84	-7.47	-62.791	-0.117	31.82	1.00
Canaliculatol-Rigid	-45.1003		-7.74	-19.875	-0.033	18.34	0.28
Discretine-Flex	-39.9325	-18.3	-6.48	-72.84	-0.23	7.127	1.97
Ecdysterone-Rigid	-37.8968		-7.97	-66.83	-0.15	6.48	1.28
Gedunin -Rigid	-40.1944		-7.77	-78.93	-0.21	-10.12	1.79
Kalopanax saponin I - Rigid	-59.6167		-8.48	-84.05	-0.15	16.51	1.29
Laudanidine-Rigid	-38.780884		-6.26	-75.22	-0.206	2.954	1.76
Laurolitsine-Rigid	-29.632555		-6.92	-59.55	-0.185	32.51	1.58
Mahmoodin-Rigid	-41.310192		-7.46	-80.99	-0.205	9.355	1.75
Mangiferin-Rigid	-40.489849		-7.31	-80.43	-0.191	27.817	1.63
Methylripariochromene A -Rigid	-39.317663		-6.85	-58.66	-0.171	11.019	1.46
Myricitrin-Rigid	-44.184696		-7.4	-59.52	-0.133	17.227	1.14
Myrigalone B-Flex	29.283272		-5.01	-34.03	-0.29	8.713	0.24
Myrigalone G-Rigid	-36.106804		-6.68	-59.33	-0.153	23.112	1.30
Nordicentrine-Rigid	-38.257423		-6.87	-75.23	-0.193	1.715	1.65
Quercetin-Rigid	-39.467415		-7.36	-56.06	-0.125	18.222	1.06
Phloretin-Flex	-44.752022	-29.21	-7.1	-70.55	-0.161	17.712	1.38
Plumbagin-Flex	-28.81204	-17.29	-6.68	-57.28	-0.198	4.611	1.69
Resveratrol-Rigid	-35.77031		-7.12	-68.92	-0.171	17.478	1.46
Catechin-Rigid	-34.71438		-7.18	-68.59	-0.205	25.9	1.75
Kaempferol-Flex	-42.17932	-20.6	-7.16	-65.82	-0.147	18.854	1.26
Myricetin-Flex	-38.58151	-18.27	-7.3	-60.89	-0.141	19.814	1.21
Thimoquinone-Flex	-27.27477	-16.61	-6.51	-47.17	-0.197	52.742	1.68
β-Caryophyllene-Flex	-23.15116	-16.21	-6.94	-29.09	-0.118	14.663	1.01
Eugenol-Rigid	-24.97930		-5.95	-59.29	-0.243	5.75	2.08
Usnic acid-Rigid	-51.692749		-7.48	-52.35	-0.129	18.33	1.10
Ascorbic acid-Rigid	-26.586088		-5.66	-46.13	-0.185	23.25	1.58

Analysis of the Hex and DOCK6 binding scores results (Tables 3 and 4), for natural compounds which have been docked to receptor β-catenin chain A, showed that the following compounds may be able to form a stable ligand-receptor complex. These compounds were Kalopanax saponin I, Canaliculatol, 1-Alpha hederin, (+)-lyoniresinol-3-a-O-ß-D-glucopyranoside, Myricitrin, Methylripariochromene A, Mangiferin, Myricetin, Discretine, Gedunin, Mahmoodin, Nordicentrine, Catechin, Myrigalone G, Laudanidine, Ecdysterone, Kaempferol, Laurolistine, Phloretin, Annonacin, Quercetin, 4-o-methyl-cyptochorophaeic acid, Calozeyloxanthone, Atranorin, Ursonic acid, 3,6-dimethyl-2-hydroxy-4-methoxybenzoic_acid and

Resveratrol. As evident from (Tables 3 and 5) Hex and DOCK6 results, the following natural compounds that have been docked with the Smo receptor showed a favourable stable binding energy. These compounds were Kalopanax saponin I, Canaliculatol, Myricitrin, Methylripariochromene A, Mangiferin, Myricetin, Discretine, Gedunin, Mahmoodin, Nordicentrine, Catechin, Myrigalone G, Laudanidine, Ecdysterone, Kaempferol, Resveratrol, Laurolistine, Phloretin, Quercetin, 4-o-methyl-cyptochorophaeic acid, Calozeyloxanthone, 3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid, Atranorin and Ursonic acid.

Table 5. DOCK6 docking analysis of selected natural compounds with the Smo receptor (Grid score, Drug Score, X score, PCS, RMSD and PCS ligand/PCS reference ligand)

Ligands	Grid score	Hawkins GB	X- score	Drug score	PCS	RMSD	PCS ligand/PCS reference
Ref: Smo with anti-tumor agent			-11.71	-207.317	-0.201		
(+)-lyoniresinol-3a-O-β-glucoside - Rigid	3838.3127		-7.35	244.032	0.199	7.834	-0.99
1-alpha-hedarin- Rigid	6527.9546		-11.85	167.583	0.109	11.208	-0.54
2-Hydroxy-p-anisaldehyde -Rigid	-25.15111		-6.51	-58.117	-0.216	5.678	1.07
3,6-dimethyl-2-hydroxy-4-methoxybenzoic_acid-Flex	-36.46623	-11.45	-7.5	-78.91	-0.202	0.942	1.00
4-o-methyl-cyptochorophaeic_acid - Rigid	148.98805		-8.24	-81.148	-0.092	2.137	0.46
Annonacin -Rigid	2129.222		-7.85	427.252	0.384	11.979	1.91
Atranorin -Flex	-65.5594	-22.34	-9.13	-123.094	-0.173	2.627	0.86
Canaliculatol-Rigid	1645.328		-10.8	-642.175	0.403	4.56	-2.00
Discretine -Flex	-29.9394	43.89	-8.7	-106.946	-0.154	6.043	0.77
Ecdysterone -Rigid	-23.8973		-11.1	-104.698	-0.117	3.355	0.58
Gedunin-Rigid	107.9163		-10.51	-62.18	-0.074	2.732	0.37
Kalopanax saponin I - Rigid	6527.9546		-11.85	-167.583	0.109	11.208	-0.54
Laudanidine-Flex	-44.72987	25.2	-8.41	-102.161	-0.137	8.874	0.68
Laurolitsine-Rigid	-54.31556		-8.58	-116.904	-0.185	1.409	0.92
Mahmoodin -Rigid	60.499504		-10.85	-87.894	-0.092	2.805	0.46
Mangiferin- Flex	-72.81403	-13.2	-9.22	-139.465	-0.169	2.496	0.84
Methylripariochromene A - Flex	-41.9596	-26.69	-8.39	-101.7	-0.191	5.084	0.95
Myricitrin-Rigid	47.06108		-9.1	-47.962	-0.051	5.363	0.25
Myrigalone B -Rigid	-19.10391		-5.21	25.399	0.046	1.328	-0.23
Myrigalone G -Flex	-50.91149	-17.24	-8.85	-114.888	-0.198	13.195	0.99
Quercetin-Flex	-55.42571	-17.98	-8.63	-102.829	-0.166	2.696	0.83
Nordicentrine -Flex	-38.78071	22.68	-8.68	-119.288	-0.186	4.868	0.93
Phloretin -Flex	-54.427528	-27.98	-8.42	-107.7	-0.202	5.496	1.00
Plumbagin-Flex	-28.469246	-20.26	-7.79	-80.351	-0.197	2.707	0.98
Resveratrol-Rigid	-45.34874		-8.37	-106.79	-0.234	1.176	1.16
Catechin -Rigid	-35.769302		-8.29	-93.002	-0.161	9.714	0.80
Kaempferol-Rigid	-52.33612		-8.52	-98.5	-0.167	4.276	0.83
Myricetin-Rigid	-49.9857		-8.56	-105.03	-0.161	2.561	0.80
Thimoquinone-Flex	-34.6884	-21.44	-7.26	-71.244	-0.217	0.925	1.08
β-Caryophyllene-Rigid	-15.6291		-8.64	-69.078	-0.182	6	0.91
Eugenol-Rigid	-16.2799		-7.14	-73.782	-0.203	2.171	1.01
Usnic acid -Rigid	-33.3885		-9.11	-84.7	-0.123	2.498	0.61
Ascorbic acid -Rigid	-29.1918		-6.35	-60.9	-0.167	2.676	0.83

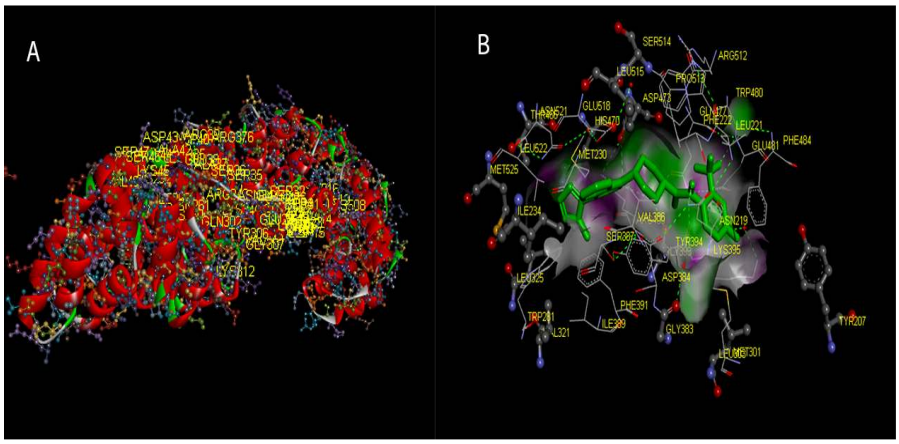


Fig. 3. Predicted docking pose of hTCF-4 at the β-catenin chain A and LY2940680 at the Smo
(A) Interaction map for hTCF-4 binding site amino acids residues of β-catenin chain A (ball and stick rendering). (B) Interaction map for ligand LY2940680 at binding site amino acids residues (ball and stick rendering) of Smo receptor

In this study the importance of total binding energy has to be considered with care, since the larger reference ligand will always release a greater total binding energy, if solely because of its size and the sheer number of contacts. The smaller natural product ligands can bind to a small region of the active site and as a result releases a smaller amount of total energy. This may be the reason why natural product binding energy values are lower than that of the reference ligand. In order to evaluate this effect, additional docking interaction analyses were carried out.

3.5 Docking Interaction Analysis

Binding patterns and interacting residues of the hTCF4 complex with β-catenin chain A and human Smo receptor complex with anti-cancer agent LY2940680 and β-catenin chain A and Smo receptor with natural compounds were studied using Accelrys Discovery Studio 4.0. The residues of β-catenin involved in the interaction with hTCF- 4 (Chain B) were His 260, Asn 261, Lys 292, Ile 296, Asp 299, Tyr 307, Lys 312, Lys 335, Arg 376, Arg 386, Asn 387, Asn 426, Cys 429, Lys 435, Cys 466, His 470, Arg 474, and Lys 508 (Fig. 3A). The residues of the Smo receptor involved in the interaction with its anti-tumor agent (Fig. 3B) were Phe 484, Lys 395, Met 301, Leu 221, Asn 219, Asp 384, Val 386, Ser 387, Ile 389, Met 230, Trp 281, Leu 522, Asn 521, Phe 391, Glu 518, Arg 400, Tyr 394, Pro 513, Gln 477, Trp 480, and Glu 481.

The natural compounds were expected to bind at the hTCF-4 binding active site residues of β-catenin chain A and the anti-tumor agent binding active site residues of the Smo receptor in order to prevent the biological activity of each of these receptor proteins in CSCs. According to Accelrys Discovery Studio 4.0 results, there were no similarities in the binding group composition of the natural compounds and the Smo receptor active site binding region of the reference ligand. However, there were similarities in the binding residues of natural compounds and the β-catenin chain A active site binding region of hTCF-4. This may be due to the large search box generated by CASTp for the Smo receptor, which includes many other interaction sites which would be occupied *in vivo* but which are not occupied *in silico* and hence competes for the ligand binding.

According to the results of docking interaction analysis, Gedunin was found to interact with β-catenin chain A forming bonds at the hTCF-4 interacting region involving residues His 260, Lys 292, Ile 296, Asp 299, Lys 335, and Arg 376 whereas Kaempferol interacted with β-catenin forming bonds at the hTCF - 4 interacting region involving residues Cys 429, Asn 430, Lys 435, His 470, Arg 474 and Lys 508. Mangiferin, Methylripariochromene A, Myrigalone G, Nordicentrine, Catechin, Myricetin, Discretine, Laurolitsine, Myricitrin, Phloretin, Canaliculatol and Ursonic acid interacted with β-catenin chain A forming bonds at the hTCF-4 interacting region involving residues Cys429, Asn 430, Lys 435, Arg 469, His 470, Arg 474 and Lys 508. Kalopanax saponin I interacted with β-catenin chain A forming bonds at the hTCF-4 interacting region involving residues His 260, Asn 261 and

Ile 296. (+)-Lyoniresinol-3a-O-β-glucoside interacted with β-catenin forming bonds at the hTCF-4 interacting region involving residues Asn 430, Lys 435, Cys 466, Arg 469, His 470, Arg 474 and Lys 508. Gedunin derivative (Mahmoodin) showed interaction with β-catenin chain A forming bonds at the hTCF-4 interacting region involving residues His 260, Asn 261, Lys 292, Ile 296, Asp 299, Lys 335, and Arg 376. Despite their relatively good scores when docked with β-catenin chain A, the natural compounds 1-alpha-hedarin, 4-o-methyl-cyptochorophaeic_acid, Annonacin, 3,6-dimethyl-2-hydroxy-4-methoxybenzoic_acid, Atranorin, Laudanidine, although, Quercetin and Resveratrol were not found to interact with the hTCF-4 relevant region residues on these detailed analyses.

3.6 Scoring

An additional analysis was carried out for selecting the better β-catenin chain A docking pose for the reference ligand and all the selected natural compounds (35 compounds), which were scored using Drug score, X score, docking-program specific scores, RMSD (Root-mean-square deviation), and PCS. Finally, combining all these scores was considered to be efficient enough to select the best docking pose. Relative affinity predictions were based on two kinds of results: General affinity (X-Score, DrugScore, docking-program specific scores), which tells which pose binds with greater strength to the target protein, and average affinity (PCS) which gives a measure of binding affinity differences irrespective of molecule size, considering the contribution of each contact [47,48].

The β-catenin reference ligand, hTCF-4 is a large polypeptide. Due to its large size, the number of possible interactions between the natural ligands and the receptor were very high. It is unlikely that a small molecule may establish so many interactions. This is evidenced when looking, e.g. at the DrugScore for the β-catenin reference ligand (-243.543), which is larger than the scores of any natural product ligand considered. However, if the small molecule can bind a small part of the active site more efficiently, then it could bind with greater affinity than the reference ligand, and hence competitively inhibit and preclude binding of the reference ligand. Therefore, comparisons should be based on binding affinity differences in the small region covered by the ligand. Since we get only the overall score for the whole molecule, we considered that binding of hTCF-4 will be driven by the average affinity of the whole molecule and compared in turn the PCS values for hTCF-4 with those of the putative competitors. As can be seen, some ligands have better PCS values (more negative) than the reference ligand (which is -0.117). This is a helpful approach when comparing small molecule drugs to a larger known ligand such as hTCF4.

Whenever any of the scores (Grid, MM-GB/SA, DrugScore or X-score) becomes clearly positive (>0), it is a good indication that the ligand is probably not bound to the active site and those poses were discarded. It is worth noting that, in the present study, there were a few cases where it was difficult to isolate a single best pose, because none of them had the best value in all scores. As shown in Table 4, these were evaluated using binding site residue analysis and the relative affinity calculation of $PCS_{\text{natural ligand}}$ / $PCS_{\text{reference}}$.

RMSD is most useful when looking for specific analogues of the known substrate [49]; otherwise, it might be misleading. RMSD was of very little value in beta-catenin since comparison with a huge polypeptide leads to large RMSD values in most cases. It may be more sensible to use RMSD in Hedgehog signaling pathway analysis (Smo receptor), although here, drugs from very different groups, sizes and with different properties are being tested.

According to score results shown in Table 4, the compounds such as 1-Alpha hederin, 3,6-dimethyl-2-hydroxy-4-methoxybenzoic_acid, 4-o-methyl-cyptochorophaeic acid, Annonacin, Atranorin, Myricitrin, Discretine, Ecdysterone, Gedunin, Kalopanax saponin I, Laudanidine, Laurolistine, Mahmoodin, Mangiferin, Methylripariochromene A, Myrigalone G, Nordicentrine, Quercetin, Phloretin, Plumbagin, Resveratrol, Catechin, Kaempferol, Myricetin, Thimoquinone, βCaryophyllene , Eugenol, Ursonic acid and Ascorbic acid have better X score, PCS, and average affinity (PCS_{ligand}/ $PCS_{\text{reference ligand}}$) with β-catenin chain A. Overall the results of this study (docking energy of Hex 8.0.0 and DOCK6 for each conformer, docking interaction residues analysis, DrugScore, X-score and PCS average affinity analysis) clearly suggested some potentially useful compounds and their comparative modes of interaction and possible inhibitory effects on the β-catenin/hTCF-4 complex in the Wnt signaling pathways of CSCs. Those are Gedunin,

Mahmoodin, Kaempferol, Methylripariochromene A, Mangiferin, Myrigalone G, Catechin, Myricetin, Discretine, Laurolitsine, Myricitrin, Nordicentrine, Phloretin, Ursonic acid and Kalopanax saponin I.

3.7 Drug Likeness Study

Drug-likeness analysis reduces the chances of selecting false positive results, such as substances that are good inhibitors but have unpleasant characters such as toxic effects, lower water solubility, lower absorption, etc. Various basic physico-chemical properties are calculated to evaluate the potential of a molecule to act as a drug [50]. Results of the present study are tabulated in Table 6. Although we identified that Mahmoodin, Kalopanax saponin I, Mangiferin and Ursonic acid as having good binding energy, interaction and binding affinity these compounds also have some properties which violate the drug likeness rules. Based on the drug likeness study Gedunin, Kaempferol, Methylripariochromene A, Myrigalone G, Catechin, Myricetin, Discretine, Laurolitsine, Myricitrin, Nordicentrine and Phloretin can be proposed as more potential drugs which can target CSCs.

3.8 Docking Analysis by AutoDock Vina

Finally, a docking analysis was carried out by using AutoDockVina for 11 selected compounds based on the results of docking, residue analysis, and drug likeness analysis to confirm them as possible β-catenin inhibitors. The results obtained from the Auto DockVina analysis are summarized in Table 7. On the basis of binding energies, the best compounds which are docked with β-catenin were Gedunin (-7.3 kcal/mol), Kaempferol (-6.1 kcal/mol), Methylripariochromene A (-5.3 kcal/mol), Myrigalone G (-5.1 kcal/mol), Catechin (-6.5 kcal/mol), Myricetin (-6.5 kcal/mol), Discretine (-5.6 kcal/mol), Laurolitsine (-5.9 kcal/mol), Myricitrin (-6.3 kcal/mol), Nordicentrine (-6.0 kcal/mol) and Phloretin (-5.4 kcal/mol). A recent in silico study carried out by Iftikhar and Rashid in 2014 has show that a sereis of experimently verified β-catenin binding flavonoid inhibitors such as isorhamnetin, fisetin, genistein and silibinin has binding energies in the range of −5.68 to −4.98 kcal/mol as assessed by molecular docking studies using AutoDock software [12,29,51,52]. However in the present study a series of novel inhibitors (Gedunin, Kaempferol, Methylripariochromene A, Myrigalone G, Catechin, Myricetin, Discretine, Laurolitsine, Myricitrin, Nordicentrine and Phloretin) exhibited binding energies in the −7.3 to −5.1 kcal/mol range. On the basis of known information [12], our finding shown considerable binding values for β-catenin inhibition. To further support, *in vitro* studies which have been carried out by other researchers proved that some of the selected compounds such as Gedunin [53], Kaempferol, Catechin, Myricetin [54], Myricitrin [55] and Methylripariochromene A [56] have anticancer activities. In addition based on the previous reports, Myrigalone G, Phloretin [57], Nordicentrine, and Discretine [35] exhibit antioxidant activity and Laurolitsine [58] exhibit anti bacterial activity.

Table 6. Properties of drug likeness analysis

Compounds	Molecular weight [g/mol]	Log P	H-bond accepter	H-bond donor	Polar surface area[Å]	Molar refractivity [cm3]	Rotatable bond count
Gedunin	482.573	4.30	7	0	95.34	125.57	3
Kaempferol	286.2363	2.64	6	4	107.22	71.4±0.3	1
Methylripariochromene A	262.301	2.12	4	0	44.76	73.78	3
Mangiferin	422.342	-0.36	11	8	197.37	96.9±0.3	2
Myrigalone G	286.3224	4.86	4	2	66.76	80.67	5
Catechin	290.271	1.80	6	5	110.38	73.6±0.3	1
Myricetin	318.235	1.85	8	6	147.68	75.02±0.3	1
Discretine	355.427	3.15	5	0	40.16	99.84	4
Laurolitsine	313.347	2.83	4	0	62.89	49.3±0.3	2
Myricitrin	464.376	0.60	12	8	206.6	106.0±0.4	3
Nordicentrine	325.358	2.63	5	1	48.95	88.69	2
Phloretin	274.268	3.90	5	4	97.99	72.9±0.3	4
Mahmoodin	526.617	3.37	8	1	112	138.62	6
Kalopanax saponin I	883.070	2.31	16	9	255	219.17	8
Ursonic acid	454.68	7.15	3	1	54	132.1±0.4	1

Table 7. Docking analysis of β catenin using AutoDockVina

Compounds	Binding energy value (kcal/mol)
Gedunin	-7.3
Myricetin	-6.5
Catechin	-6.5
Myricitrin	-6.3
Kaempferol	-6.1
Nordicentrine	-6.0
Laurolitsine	-5.9
Discretine	-5.6
Phloretin	-5.4
Methylripariochromene A	-5.3
Myrigalone G	-5.1

4. CONCLUSIONS

A large number of docking poses were evaluated on the basis of binding energy and conformations, commonly interacting residues at the binding pocket, binding affinity and drug-likeness calculations. Based on the overall results, it could be proposed that Gedunin, Kaempferol, Methylripariochromene A, Myrigalone G, Catechin, Myricetin, Discretine, Laurolitsine, Myricitrin, Nordicentrine and Phloretin may act as useful inhibitors against the Wnt signaling pathway and can be used as potential natural anti-cancer stem cell drugs. Further studies of the proposed inhibitors need to be carried out to explore their binding and inhibitory potential in *in vitro* studies. In addition, the pharmacophore model presented can be used to screen more compounds and will thus be helpful in finding novel inhibitors of β-catenin that interrupt the Wnt signaling pathway in CSCs.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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