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Research Article

In-silico Pharmacokinetic, Molecular Docking and Molecular Dynamics Simulation Studies of Phytochemicals Isolated from *Cascabela thevetia* as Potential Anticancer Agents

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ABSTRACT

Cascabela thevetia, generally recognized as yellow oleander, lippold or lucky nut (in English) and *peeli kaner* (in Hindi), is indigenous to Central America and Mexico and widely distributed in the Indian subcontinent as well, is a medicinally important herb that has long been employed to treat disorders like ulcers, scabies, hemorrhoids, and tumor dissolution. Seven cardenolides (Compounds 1-7) isolated from fruits of *C. thevetia* are selected as ligands for *in-silico* pharmacokinetic (ADME properties) and drug-likeness studies via the Swiss ADME online server. Further, molecular docking studies were accomplished for the selected ligands via the Auto Dock Vina module of PyRx to explore the receptor-ligand interaction with β-tubulin (a potential target for anticancer drugs, PDB ID: 1SA0), to find out potential therapeutic ligands for cancer chemotherapy. From the studies performed it was found that most of the ligands (compounds) have the capability of good binding affinity with the receptor, β-tubulin, good drug-likeness and ADME properties among which compounds 3, 6 and 7 are the best drug candidates because they followed the Lipinski rule of 5 with 0 violation and also have good binding affinities (-8.3, -8.0 and -7.9 kcal/mol, respectively) against the target protein. Hence, compounds 3, 6 and 7 can be developed as novel therapeutic agents in cancer chemotherapy. Further, a molecular dynamics (MD) simulation study of best-docked ligand 3 was performed to establish its stability and validate molecular docking results. Based on simulation results, it may be stated that ligand 3 was firmly attached to the receptor protein during MD simulation since none of the conformations of the receptor-ligand complex were unstable, and no folding or unfolding of the complex took place. Therefore, compound 3 can be considered a good inhibitor of β-tubulin after validation of various parameters of drug discovery.

Introduction

According to the International Agency for Research on Cancer (IARC) report 2022 from the World Health Organization (WHO), cancer is one of the prominent causes of mortality worldwide, accounting for 9.7 million losses in 2022 and 20 million new cases in 2020. By 2050, it is anticipated that there will be 35 million new instances of cancer worldwide, with low- and middle-income countries being the most severely affected. This underscores the need for enhanced healthcare infrastructure and affordable cancer services.[1] The yearly economic drain of cancer treatment was estimated to be close to US$ 1.16 trillion in 2010.[2]

Cancer is defined scientifically as an abnormal growth of cells with an inclination to proliferate uncontrollably and, in some cases spread in the whole body (metastasis).[3] Currently used cancer treatments include chemotherapy, stem cell transplant, targeted therapy, hormone therapy, immunotherapy, radiation therapy, and surgery (including cryosurgery, lasers, photodynamic therapy,

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and hyperthermia). As stated by the Chinese National Cancer Center, the persistence rate of cancer patients has amplified to 40.5% from 10% in the last decade. This astonishing consequence could be attributed to the emergence of multiple therapies for cancer.[4]

While chemotherapy and therapeutic medications have made momentous progress in the management of cancer, there is always a necessity to find new and safe anticancer therapies. Owing to the drawbacks of synthetic drugs, such as their high toxicity and adverse side effects, natural therapies prepared from medicinally important plants have become more significant in the fight against cancer. An assessment of about 14,000 extracts from nearly 35,000 plant samples was performed by the National Cancer Institute (NCI) of the US against many tumor cell lines. Out of 92 drugs for cancer treatment that were in the market in the United States before 1983 and were approved globally between 1983 and 1994, more than 62% have natural origins.[5]

The plant family Apocynaceae, which includes the genera *Thevetia*, *Apocynum, Nerium*, *Strophanthus*, and *Catharanthus* is widely known for its anticancer properties. Scientifically *Cascabela thevetia* is also known as *Thevetia peruviana (Pers.), C. peruviana* (L) or *K. Schum.*[6] *C. thevetia* is generally recognized as Lippold, lucky nut or yellow oleander (in English) and *Peeli Kaner* (in Hindi). South and Central America, particularly Brazil, Mexico, and the West Indies are the natural habitats of the plant, but it is now widely grown as a decorative plant across hot and humid regions of India and Sri Lanka as well. Traditional uses of the *C. thevetia* plant include the treatment of skin tumors, heart diseases, and gastrointestinal and inflammatory disorders.[7]

Numerous phytochemicals, such as glycosides, alkaloids, saponins, tannins, flavonoids, fixed oils and fats, and phenolic compounds, are found in yellow oleander. *C. thevetia* produces milky sap or latex, which contains a glycoside thevetin. Although thevetin is used as a cardiac stimulant, it is highly toxic in its natural state. Thevetin is a cardenolide that exists in two forms: Thevetin A and thevetin B; other forms of thevetin are neriifolin, thevetoxin peruvoside, and ruvoside. The plant seed possesses a higher level of toxicity in its inherent state compared to other plant components because its seeds have the most extensive and diverse composition of cardiac glycosides (the percentage of cardiac glycosides in fruit is 0.045%, in leaf 0.07%, in milk 0.036% and kernel 4.8%). Several cases have been reported where humans have been intentionally or unintentionally poisoned by consuming fruits and leaves.[8] While the consumption of approximately 10 fruits can be lethal for an adult and for a child, a single fruit can be deadly poisonous. The prominent symptoms commonly observed in cases of poisoning include nausea, vomiting, stomach discomfort, diarrhoea, dysrhythmias, and hyperkalemia.[9] Apart from these poisonous properties of the fruits of *C. thevetia*, the plant has tremendous anticancer properties against a wide array of cancers. Ramos-Silva and co-workers in. 2017 observed that the methanolic extract of *C. thevetia* showed cytotoxic effects on four types of human cancer cells: prostate, breast, colorectal, and lung.[10] While previous studies have assessed the anticancer properties of various components of the *C. thevetia* plant, including bark, leaves, and seeds, against human pancreatic cancer and gastric cell lines, the therapeutic efficacy of *C. thevetia* fruit remains uncertain due to the limited identification and examination of cardiac glycosides as cytotoxic agents.

Traditional drug discovery is a very tedious task that requires more than a decade and costs an average of USD 1.8 billion to bring a drug molecule into the market for the management of a disease. The perspective of *in-silico* approach to drug design to decrease the cost, time, and labour involved in drug discovery has attracted a lot of interest recently. Numerous novel medicinal compounds have been effectively advanced using computational techniques of drug design and development. In computer-aided drug discovery (CADD), also acknowledged as *in-silico* drug discovery, computers are effectively used to screen a large number of chemical compounds to find suitable drug candidates. The interaction of these compounds with appropriate drug targets is studied before *in-silico* evaluation of pharmacokinetic and pharmacodynamic properties of drug candidates.[11] A good drug candidate should have suitable ADME characteristics at a therapeutic dose, a robust interaction, and sufficient efficacy against the therapeutic target. Regarding this, various computational techniques such as drug-likeness estimation, ADME studies, molecular docking, etc., are valuable tools in identifying potential drugs/molecules from databases. These approaches can significantly reduce the time and costs associated with experimental drug discovery.[12] Using molecular docking, one can accurately forecast the mode of the interactions of the receptor-ligand complex along with the binding energy involved in it. Additionally, ADME assessment is a helpful tool for establishing the pharmacokinetic properties (such as absorption, distribution, metabolism, and excretion) of potent molecules.[13]

The eukaryotic cytoskeleton is composed mostly of microtubules, actin filaments, and intermediate filaments. Because they provide shape to cells, microtubules are essential for their proper functioning such as cell division by forming the mitotic spindle and intracellular transport. At the microtubule nucleation site, macromolecules are primarily composed of ϒ-tubulin and α, β-tubulin heterodimers. Since tubulin has become a prominent target in drug discovery, tubulin inhibitors have attracted a lot of interest as anticancer agents.[14] Many anticancer drugs such as vinca alkaloids, paclitaxel, docetaxel etc. have been developed by targeting tubulins.[15]

This work aims to evaluate seven cardenolides that have better ADME properties through *in-silico* studies. Further, a molecular docking study was executed to investigate the receptor-ligand interaction with β-tubulin (PDB ID: 1SA0) to identify possible therapeutic ligands for cancer chemotherapy. Additionally, an molecular dynamics (MD) simulation study was carried out to authenticate the outcomes of receptor-ligand binding interactions obtained by molecular docking studies.

Materials And Methods

Preparation of Ligands

The seven cardenolides (1- 7) (Fig. 1) isolated from fruits of *C. thevetia* have been selected as ligands for the *in-silico* studies whose cytotoxic activities on different cell lines have already been reported. [16] Chemdrwa Ultra 12.0 has been employed to draw the structures of ligands which were then changed to 3D structures and saved in sdf format by using Marvin Sketch software. The energy of the ligands was minimized by applying the MMFF94 force field in the PyRx. [17] Lastly, energy-minimized ligand molecules were converted into pdbqt format via Auto Dock of PyRx and used for further molecular docking investigations on PyRx.

Preparation of Proteins

The 3D structure of γ-tubulin (PDB ID: isa0) with co-crystallized ligand was taken from the protein data bank of RCSB. Biovia Discovery Studio 2021 and UCSF Chimera 1.16 software were used for further preparation of proteins for molecular docking. For molecular docking, the B chain of β-tubulin was selected since its active site is present in this chain. Using Auto Dock tools, the co-crystallized ligand was detached from the protein structure, and polar hydrogens, water molecules and charges were affixed.[18] Finally, pdbqt file of the protein was obtained and used for molecular docking investigations.

Molecular Docking

Molecular docking studies were accomplished via the Auto Dock Vina module incorporated in PyRx. The energy-minimized protein structure was converted to an Auto Dock macromolecule in PyRx. The energy-minimized protein and ligands were added in Vina Wizard to run a docking study.[17] The active sites of target proteins were determined based on how they bind to their natural ligands (in this case Colchicine). The input grid box has dimensions 36.83 × 30.34 × 36.99 Å and the center at 103.44 × 80.32 × 1.62 Å were integrated into Vina workspace.

In-silico Pharmacokinetic Study

The pharmacokinetic studies of the ligands 1-7, were performed by using the Swiss ADME online server. For evaluating the pharmacokinetic properties of compounds, first of all, structures were sketched on Chem Draw Ultra-12 and smiles were generated via Open Bable. The smiles of ligands were then uploaded at the interface of the Swiss ADME website and a Swiss drug design command was run to generate ADME characteristics of ligands with acceptable parameters. The Lipinski filter was operated to envisage the ligands based on their drug-likeness criteria including lipophilicity, water solubility, total polar surface area (TPSA), gastro-intestinal (GI) absorption, blood brain barrier (BBB) permeability, and skin penetration.[19,20] The brain or intestinal estimated permeation method (BOILED-Egg model) was employed to deduce the ADME and druglikeness properties of compounds. The GI absorption and BBB penetration of the compounds are shown in the figure of a BOILED-Egg. This model helps to determine the lipophilicity and polarity of molecules. The yellow area in the BOILED-Egg representation denotes a zone of high possibility of BBB permeability, while the white zone represents GIT absorption. The red dots present in the figure designate that the molecule is not affected by P-glycoprotein-assisted extrusion of the central nervous system. The analysis also envisages whether or not the compounds will exhibit good GI absorption.[21]

MD Simulation Analysis

After the molecular docking assessments, the highest-scoring complex was put through molecular dynamics simulations using Schrodinger's Desmond tool to determine its stability and validate the docking results.[22] A POPC membrane was used to prepare the system after the docked complex was imported into a project table. The solvated model was SPC solvent model housed in an orthorhombic box. To maintain physiological conditions, a salt strength of 0.15 M was employed. The energy reduction for the study was evaluated by applying the hybrid techniques steepest decent and the 'Limited-memory Broyden–Fletcher–Goldfarb–Shanno' (LBFGS) algorithms for 2000 iterations. A 'Nose-Hoover' chain

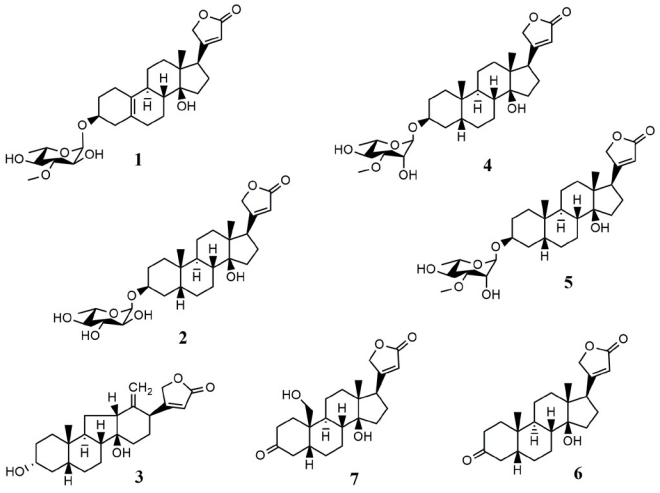

Fig. 1: Structure of cardenolides selected as ligands

thermostat operating at 300 K temperature and pressure of one bar for 100 ns in combination with an isobaric isothermal ensemble (NPT equilibrium) was used to accomplish the simulation. The trajectory of the simulation was examined utilizing the interaction diagram employing a variety of factors, for instance, the root mean square deviation (RMSD), the radius of gyration (Rg) of the complex, the interfering hydrogen bonds and the root mean square fluctuation (RMSF).

Table 1: Ligand-receptor interaction data of cardenolides against β-tubulin (PDB ID: 1SA0) using PyRx tool for virtual screening

Compound No.	*Binding affinity (in kcal/mol)*	*Amino acids interactions with the shortest bond lengths* **Amino acids with conventional H-bond* *Others are hydrophobic interactions*	*Distance (in Å)*
1	-7.7	ARG390B* ALA298B	2.54 3.48
2	-5.2	PRO175B* TRY224B* LYS176B	2.21 2.29 5.10
3	-8.3	GLU386B* ALA304B ASP211B	2.05 5.08 2.91
4	-7.9	LYS389B* CYS305B* ALA298B ALA304B ASP211B	2.65 1.36 3.91 4.41 2.91
5	-7.8	ARG215B* GLU386B* LYS389B ARG390B	3.36 3.57 4.12 4.19
6	-8.0	LYS254B	3.66
7	-7.9	ASP211B* ALA298B	2.29 3.60
Colchicine	-5.7	ARG290B ARG290B ALA304B ALA304B ALA304B	3.73 4.13 4.83 4.38 5.09

Results And Discussion

The findings of molecular docking and pharmacokinetic studies are tabulated in Tables 1 and 2, respectively. Molecular docking of selected ligands was achieved via the Auto Dock Vina module of PyRx against β-tubulin (PDB ID: 1SA0). The BOILED-Egg model was employed to calculate the ADME and drug-likeness properties of the ligands by the Swiss ADME online server. 'Lipnski's rule of 5' was operated to estimate the drug-likeness properties of the ligands. After the molecular docking and drug-likeness assessments, the highest-scoring complex with ligand 3 and β-tubulin was put through molecular dynamics simulations using Schrodinger's Desmond tool to determine its stability and validate the docking results. The results of docking investigations are related to the interactions between target protein and ligands. The number of H-bonding interactions, binding affinity scores, and other hydrophobic interactions were used for establishing the outcomes of the protein-ligand bonding interactions. The negative binding affinities indicate that the receptor and ligand are in stable binding interactions.

Molecular Docking Results

A technique that is becoming more and more important for realizing the basis of protein-ligand interaction is molecular docking.[23] This present study was accomplished to determine the binding affinities of the ligands with the groups present in the active site of β-tubulin (PDB ID: 1SA0) shown in Table 1. The degree of the interaction between the ligand and the protein is

Table 2: Pharmacokinetic and ADME calculations of ligands by Swiss ADME

Compound No	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Formula	$C_{29}H_{42}O_8$	$C_{29}H_{44}O_8$	$C_{23}H_{32}O_4$	$C_{30}H_{46}O_8$	$C_{30}H_{46}O_8$	$C_{23}H_{32}O_4$	$C_{23}H_{32}O_5$
Mol. Weight (g/mol)	518.64	520.65	372.50	534.68	534.68	372.50	388.50
iLOGP	3.87	3.85	3.08	3.48	3.98	3.32	2.59
Water solubility	Soluble	Moderately soluble	Soluble	Moderately soluble	Moderately soluble	Soluble	Soluble
GI absorption	High	High	High	High	High	High	High
BBB penetration	No	No	Yes	No	No	Yes	No
P-glycoprotein substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Cytochrome P450 Inhibitor	No	No	No	No	No	No	No
Log Kp (cm/s) epidermis Penetration	-8.77	-7.98	-6.83	-8.07	-8.07	-7.00	-7.96
Druglikeness (Lipinski's rule of five)	Yes; 1 violation: MW>500	Yes; 1 violation: MW>500	Yes; 0 violation	Yes; 1 violation: MW>500	Yes; 1 violation: MW>500	Yes; 0 violation	Yes; 0 violation

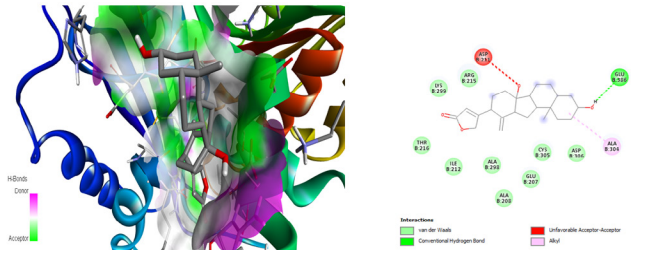

Fig. 2: Display of H-bond on receptor surface and 2D interactions of Compound 3

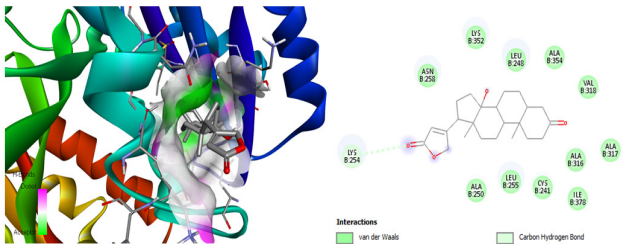

Fig. 3: Display of H-bond on receptor surface and 2D interactions of Compound 6

indicated by the binding affinity of the protein-ligand complex. The binding affinity of the reference compound, Colchicine, the natural ligand of β-tubulin has shown the value of -5.7 Kcal/mol when docked with receptor and have zero conventional H-bond with amino acids whereas hydrophobic interactions were detected with amino acids ARG290B and ALA304B. The docking results showed that ligands 1-7 have better binding affinities greater than -7.7 Kcal/mol except compound 2 (-5.2 Kcal/mol) than Colchicine. Among them, the binding affinities of compounds 3 and 6 have come out as the highest (-8.3 and -8.0 Kcal/mol respectively). Compounds 1 (-7.7), 4 (-7.9), 5 (-7.8), and 7 (-7.9) have also good binding affinities (written in parentheses in Kcal/mol) against the target protein. Compound 1 interacts through one conventional H-bond with amino acid ARG390 (bond distance 2.54 A^0) along with other interactions with ALA298B. Compound 2 interacts with protein through two conventional H bonds with amino acids PRO175B and TRY224B (bond distance 2.21 and 2.29 A^0 respectively) and other hydrophobic interactions with LYS176B. Compound 3 interacts through one conventional H-bonding with amino acid GLU386B (bond distance 2.05 A^0) and the hydroxyl group present in the compound. It interacts with amino acids ALA304B and ASP211B through hydrophobic interactions. Compound 4 forms two hydrogen bonds with amino acids LYS389B and CYS305B with bond lengths equal to 2.65 and 1.36 A^0 respectively and also it interacts with ALA298B, ALA304B and ASP211B through other interactions. Compound 5 forms two hydrogen bonds with amino acids ARG215B and GLU386B with bond lengths equal to 3.36 and 3.57 A^0 respectively and also it interacts with LYS389B and ARG390B through other interactions while compound 6 fits well into the binding pocket of the protein through hydrophobic interactions with amino acid LYS254B. Compound 7 forms one conventional H-bond with ASP211B 9 (bond distance 2.29 A^0) and one hydrophobic interaction with ALA298B. Compounds 1, 2, 4 and 5 contain sugar moiety connected with glycosidic linkage and exhibit good binding affinities with the target protein except compound 2. Compounds 3, 6 and 7 contain no sugar moiety and show the best docking results whose hydrogen bonding on the surface of receptor and 2D interactions are shown in Figs 2-4 respectively.

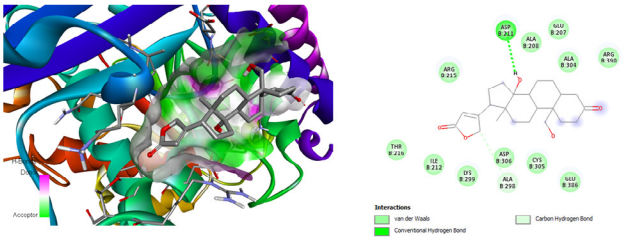

Fig. 4: Display of H-bond on receptor surface and 2D interactions of Compound 7

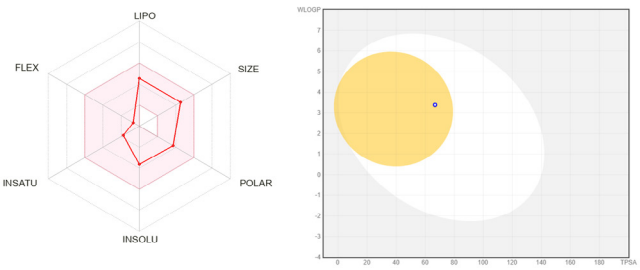

Fig. 5: Left: Oral availability prediction chart: A coloured zone is a suitable physicochemical space for oral bioavailability. Righ: Boiled egg plot: to show the highest possibility of being absorbed by the gastrointestinal tract [For Compound 3]

SwissADME results

'Swiss ADME Predictor' is a computer-based programme dedicated for calculating the pharmacokinetic characteristics and qualities of drug-like chemical entities based on their molecular structures.[24] The compounds that lie in the range of the pink area of the RADAR plot are ingestible. ADME properties of all compounds 1-7 as shown in Table 2. Oral bioavailability radar and boiled egg plot for interpretation of gastrointestinal absorption of compounds 3, 5 and 7 are depicted in Figs 5-7 respectively. They are moderately to highly soluble in water and are anticipated to absorb well via the gastrointestinal tract. Only two compounds 3 and 7 were established to be BBB permeable. All the compounds were predicted as P-glycoprotein substrates and not as inhibitor cytochrome P450. It was estimated that all explored compounds would be skin permeable because of their high Log Kp values. Lipinski's rule of 5 was employed to evaluate the results of

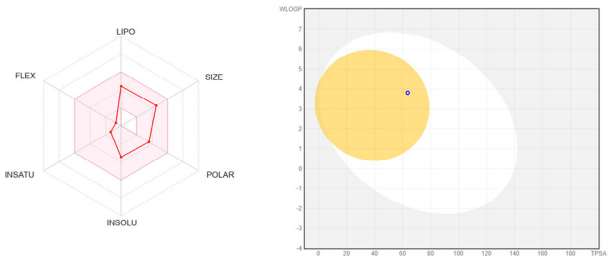

Fig. 6: Left: Oral availability prediction chart: A coloured zone is a suitable physicochemical space for oral bioavailability. Righ: Boiled egg plot: to show the highest possibility of being absorbed by the gastrointestinal tract [For Compound 6]

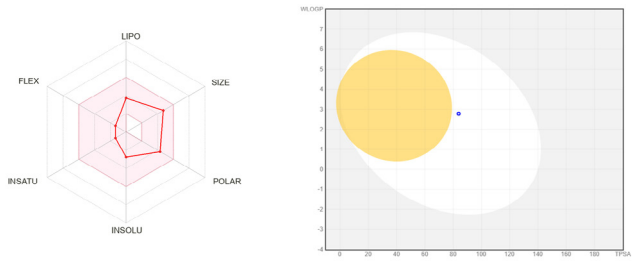

Fig. 7: Left: Oral availability prediction chart: A coloured zone is a suitable physicochemical space for oral bioavailability. Righ: Boiled egg plot: to show the highest possibility of being absorbed by the gastrointestinal tract [For Compound 7]

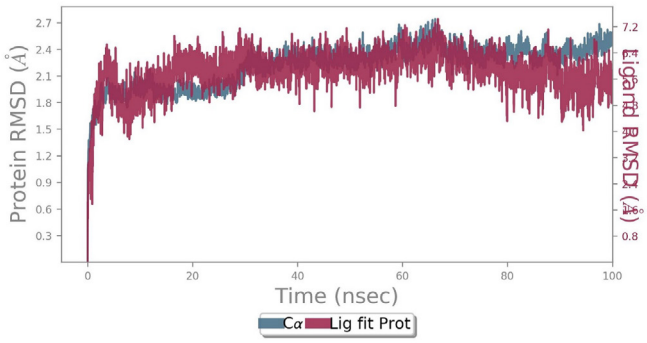

Fig. 8: RMSD graph of the docked complex formed between Compound 3 and target protein β-tubulin (PDB ID: 1SA0)

ADME properties. Compounds 3, 6 and 7 have 0 violations from this rule and the rest of the compounds (1, 2, 4, and 5) have 1 violation from the Lipinski rule, which for orally active medicines is acceptable. [19] Since all the compounds follow the Lipinski rule of 5 with 0 or 1 violation, so all of them can act as efficient orally active drugs. Thus from the above results, we may conclude that most of these compounds have good drug-likeness and ADME properties among which compounds 3, 6 and 7 are the best drug candidates because they follow the Lipinski rule of 5 with 0 violation and also have good binding affinity against the target protein.

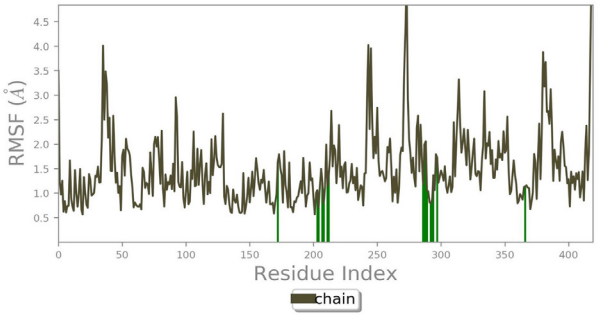

Fig. 9: RMSF graph of the docked complex formed between Compound 3 and target protein β-tubulin (PDB ID: 1SA0)

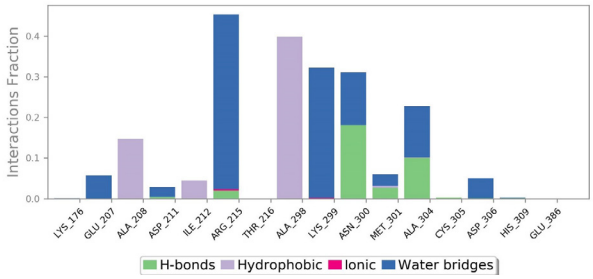

Fig. 10: Protein-ligand interaction diagram of the docked complex formed between Compound 3 and target protein β-tubulin (PDB ID: 1SA0)

MD simulation studies

MD simulations of the best-docked complex formed between compound 3 and target protein β-tubulin (PDB ID: 1SA0) were used to determine the intensity of the compound's binding to its associated target protein. Fig. 8 shows the RMSD graph for the simulated complex. For the docked complex RMSD was found to be 2.3 Å. The protein and ligand are well-coped in the binding pocket. According to the RMSF plot the *C*- and *N*- terminals of the protein vary more because they are often more stiff than the protein's unstructured regions. Parts of the secondary structure, like beta strands and, alpha helices change less than loop sections of the protein (Fig. 9). Moreover, the protein-ligand interaction diagram of the docked complex is given in Fig. 10. Based on simulation outcomes, it can be revealed that the ligand and protein interacted well during the simulation since all the conformations of the complex were stable, and no folding or unfolding occurred.

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