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RESEARCH ARTICLE

Virtual screening of compounds presents in *Azadirachta indica* (Neem) seed that can interfere with the targets of SARS-CoV-II viral entry, viral RNA replication, and post-translational modification of viral proteins

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ABSTRACT

The novel coronavirus disease "COVID-19" has affected almost 5.2 million people and approximately 337,000 peoples have deceased because of the pandemic. There is necessity for the discovery of different drug or vaccines for the management of this virus. We have selected 20 compounds that are present in neem seed, majority of which are limonoids. Limonoids are highly oxygenated modified triterpenes that are well known for their antiviral (HIV), antibacterial, anticancer, antimalarial, antifungal, and pesticidal activity. The objective of this study is to evaluate the natural compounds present in neem seeds as promising inhibitors of severe acute respiratory syndrome coronavirus II (SARS-CoV-II). The study has been done by molecular docking using Autodock Vina software and the targets that are chosen for the study are main protease of SARS CoV-II main protease, RNA dependent RNA polymerase, spike protein, and human angiotensin-converting enzyme II. The binding energies of these natural compounds have been calculated in comparison with traditional drugs such as lopinavir, remdesivir, and the most debated one, that is, hydroxychloroquine. The compounds azadirachtin D and azadirachtin H, azadiradione, epoxyazadiradione, gedunin, nimbidiol, salannin, salannol, desacetylgedunin, and azadirone showed good binding energies. Absorption, distribution, metabolism, and excretion analysis of these compounds showed that the compounds also possess good solubility, absorption property as well as no toxicity. The best candidate molecule for each target has been undergone for molecular dynamics (MD) simulation study and MM-PBSA study for free energy calculation. MD simulation study showed that the drugs have huge impact on the corresponding protein structures. In MM-PBSA study, it has been found that all the compounds possess good binding energy toward their corresponding targets. Our study concluded that these natural compounds may be used for treatment of coronavirus disease 2019 and also to prevent the entry of SARS-CoV-II into host cell. Further experimental study is required for the confirmation of inhibition activity of these potential candidates against the virus.

KEY WORDS: *Azadirachta indica*, Molecular Docking, SARS-CoV-2, and Molecular Dynamics

INTRODUCTION

The coronavirus disease 2019 (COVID-19) has been announced as pandemic worldwide by World Health Origination (WHO) in mid-February 2020. According to the WHO, there are almost 4 million of people affected worldwide by the COVID-19 (WHO Coronavirus Disease

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[COVID-19] Dashboard), which leads to more than 250 thousand death across the world. The novel coronavirus also known as SARS-CoV-II was diagnosed in late December of 2019 and early January of 2020 in Wuhan city of China (Zhou *et al*., 2020; Wu *et al*., 2020). The virus was declared as novel "severe acute respiratory syndrome coronavirus II" (SARS-CoV-II) by international committee on taxonomy of virus (Coronaviridae Study Group *et al*., 2020) and has been named on the virus which led to SARS outbreak in 2003. These two viruses are genetically similar to some extent but new SARS-CoV-II virus also has similarity to HIV. It is spreading continuously across the worldwide, the most affected countries are USA, Italy, Spain, France, Germany, Brazil, Russia, and now India has reached the fourth place in confirmed cases. The first case of COVID-19 was diagnosed in India at the end of January. Almost 6,05,220 active cases are present in India and 17,848 deaths has been observed according to the official data of ministry of health and family welfare (Ministry of Health and Family Welfare Government of India). Unfortunately, there is no notable treatment of this disease till date, the management was based on symptoms of the affected patient. However, some efforts have been made for the development of diagnostic kits, therapeutics, and vaccines for the treatment of COVID-19. Apart from this, few drugs such as hydroxychloroquine, remdesivir showed some effectiveness during *in vitro* experiments. Although the clinical trial results of these drugs are not very beneficial and had several adverse effects (https://cen.acs.org/pharmaceuticals/drug-development/Coronavirus-puts-spotlight-chloroquine-questions/98/i12). This evoked us to study several important natural compounds on COVID-19 as many compounds of plant origin have been shown to possess promising activities that could assist in the prevention and/or amelioration of many severe diseases from contagious diseases like AIDS or non-contagious like Cancer. Due to several adverse effects of the synthetic entities, nowadays, many therapeutics are natural products orientated. The natural products are used due to various reasons such as fewer side effects, less cost, and long-term benefits.

The CoVs are a large family of single-stranded RNA viruses with varying sizes of 26kb- 32kb in length. Coronaviridae family is categorized into four types consisting of α- and β-CoV is known to infect mammals, δ-, and γ-CoVs infect birds. Coronavirus associated severe acute respiratory syndrome, that is, SARS (2002) and the Middle East respiratory syndrome i.e. MERS (2012) were β-CoV related outbreaks and resulted in nearly 800 dead (Sinha *et al*., 2020).

Repurposing of the drugs has been used now a days for the identification of potential drugs (Aati *et al.* 2019). Significant efforts have been made to reuse food and drug administration (FDA) approved, drugs in clinical trials for coronavirus disease (Fletcher *et al.* 2020).[59] Symptoms such as fever, fatigue, sputum production, respiratory shortness, sore throat, headache, diarrhea, and vomiting were on the rise as pneumonia cases became apparent since December 2019 and later in Wuhan, China was identified as β-coronavirus.[60] Scientists seek to figure out why SARS-CoV-II-infected populations are experiencing rising infection and death. China's new data based on analysis of confirmed cases signified, the elderly population and those with prevailing severe diseases are at higher risk for COVID-19. The mortality rate of healthy individuals from COVID-19 is below 1%, 7.3% for diabetic, 6% for chronic respiratory disease, and stands at 10% for people suffering from cardiovascular diseases.[61] For some unexplained reasons, these viruses can cross species barriers and may cause illness in humans, ranging from the cold symptoms to more severe diseases like SARS. Interestingly, the coronavirus more likely has originated from bats after which moved into other mammalian species just like the Himalayan palm civet for SARS-CoV, and of course the dromedary camel for MERS-CoV and then entered into human species (Cyranoski *et al.* 2020, Gorbalenya *et al*. 2020). The dynamics of SARS-CoV-II is not confirmed till now, but due to similarity with coronaviruses from bats (Report of the WHO-China Joint Mission on COVID-19 (PDF) (Report). This virus has round or elliptic and often pleomorphic form, and a diameter of approximately 60–140 nm (Cascella *et al*., 2020). Like other CoVs the virus uses a special surface glycoprotein called a "spike" (SARS-2-S) to connect and enter the host cell. Spike glycoprotein engages angiotensin-converting enzyme II (ACE II) as its entry receptor and uses the cellular serine protease TMPRSS2 for S protein priming (Babadaei et al. 2020, Boopathi *et al.* 2019).

Medicinal plants are principal origins for novel medicine that produces the part of traditional medicine, nutraceuticals, folk medicines, food supplements, and intermediates of pharmaceuticals and formation of leads molecules in synthetic medicine. According to the WHO, 80% population of the world used the plants origins for their daily health-care necessity. Overutilization of these medicinal plants can lead to depletion of number in the biodiversity and put these plants in endangered category (Nayar and Sastry, 1987). Neem is very important medicinal plant in Ayurveda, Homeopathic, and Unani system of medicine (Quraishi *et al*., 2018). The neem tree is used in ancient Indian medicine for 4500 years and is commonly known as village pharmacy due to its high therapeutic value (Giri *et al*., 2019). Neem scientifically referred as *Azadirachta indica* is popular medicinal tree native to Indian subcontinent. It is referred by different names such as imba, Indian lilac, limba, limbo, mambo, margosa, nim, nimbi, nimmi, nimbhagaha, vepa, and many more (Ezzat *et al*., 2018).The significance of the neem was acknowledged by US national academy of science by releasing a report with title "Neem- a tree for solving global problems"(Biswas *et al*., 2002). Neem was accepted

by the US environment protection agency for use as food crops and studies showed safe to use toward animals, humans and all insects (Bhowmik *et al*., 2010). It is an evergreen tree favourably grows up to a height of 20–35 m. Neem tree grows naturally and also cultivated in the states of Andhra Pradesh, Bihar, Delhi, Gujarat, Maharashtra, Orissa, Tamil Nadu, Uttar Pradesh, and West Bengal (Ezzat *et al*., 2018).

Various parts of tree such as fruits, seeds, barks, leaves, and roots have been used in preparation of various extracts with great medicinal value (Ezzat *et al*., 2018). Neem contains that various chemical constituents include nimbin, nimbolides, nimbidin, and important limonoids (such as azadiradione, epoxyazadiradione, azadirone, salannin, and gedunin). Many biologically active compounds were also extracted from neem such as alkaloids, carotenoids, flavonoids, steroids, and triterpenoids (Hashmat *et al*., 2012). Neem is used in cosmetics, pest control, in the treatment of chicken pox, fungal infection, and neuromuscular pains. It is having analgesic, antibacterial, antiulcer, antifilarial, antifertility, antihyperglycemic, anti-inflammatory, antiviral, antimalarial, antipyretic, antispasmodic, and antitumor activities (Hashmat *et al*., 2012). After publishing first modern paper on antiviral activity of neem extract in 1969 by Rao *et al*. a group of reports, it has confirmed the activity of neem extracts on different viruses such as polio virus, coxackie B group virus, HIV, and dengue virus at early step of viral genome replication (Badam *et al*., 1999; Parida *et al*., 2002; Rai *et al*., 1972; Rao *et al*., 1969; Reddy *et al*., 1974; SaiRam *et al*., 2000). Neem bark extract (NBE) inhibited the herpes simplex virus-1 (HSV-1) at the stage of viral entry in the natural target cells. After pre-incubation of HSV-1 virus with NBE, viral attachment and entry process into the target cells were blocked significantly (Tiwari *et al*., 2010). A fraction of neem seed kernel inhibited the expression of virus protein and significantly reduced the number of plaques of duck plague virus.[52] Neem seed extract also possesses anti-inflammatory effect.[53] A major type of compounds present in neem seed extracts are limonoids. Limonoids are highly oxygenated modified triterpenes[54] that are well known for their antiviral (HIV), antibacterial, anticancer, antimalarial, antifungal, and pesticidal activity. We have selected 24 natural compounds [Table 1] obtained from neem seeds most of which are limonoid and assessed their inhibition activity toward SARS-CoV-II main proteases, RNA dependent RNA polymerase (RdRp), spike glycoprotein, and its target for entry into the cell, that is, ACE-II [Figure 1].

METHODOLOGY

Preparation of ligand and protein structure

For this study, we have selected 20 compounds which are present in neem seed and most of them have previous reports of antimicrobial or anticancer activity [Table 1]. To perform the ligand-protein interaction, we re-deemed the "sdf" file of ligands or compounds from the PubChem (National Library of Medicine) and converted this sdf file into "pdb" file using Chimera (ver 1.12) software.[51] The crystalline 3D structures of proteins of COVID-19 main protease (M^{pro}) with ligands (PDB ID-6LU7), SARS-Cov-II RdRp (RNA dependent RNA polymerase) with complex of cofactors (PDB ID-6M71), Spike glycoprotein (PDB ID-6VXX), and angiotensin converting enzyme 2 (PDB ID-6LZG) were retrieved from the RSCB protein data bank (www.rscb.org). The structure of protease was cleaned by removing all water molecules, added hydrogen molecules, added the kollman charges, and by removing hetero groups using UCSF Chimera and AutoDock tools package.

Procedure for molecular docking of ligand with protein

The molecular docking has been performed using Autodock Vina tool (Trott *et al*. 2010) [46] for analyzing the ligand-protein binding with active sites. We prepared different grid file of all protein structures to confirm the binding residues for random docking. The Vander Waals forces, polar binding, and docking site of protein-ligands were analyzed and calculated using Discovery studio visualizer for evaluation.

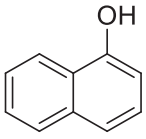
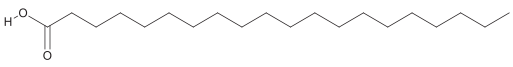
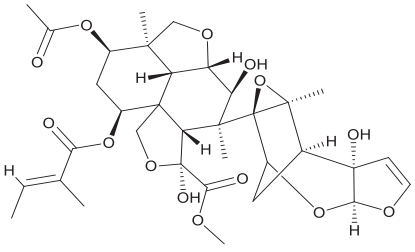
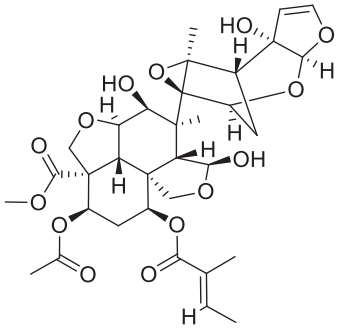
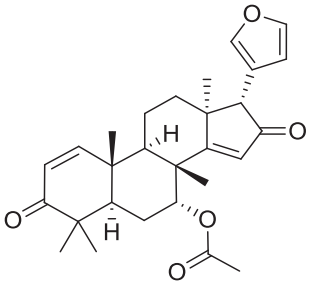
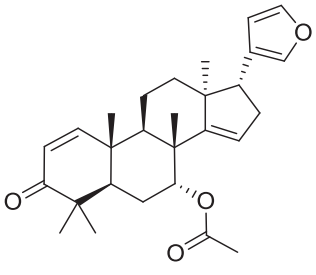
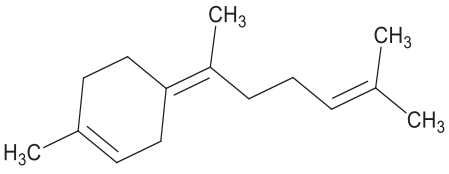
Prediction of absorption, distribution, metabolism, and excretion (ADME)

ADME studies are crucial for determination of the pharmacodynamic parameter of the selected molecules or drug. We selected pkCSM database[47] to analyse the ADME parameter of the selected compounds of neem seeds which showed good binding affinity toward COVID-19 targeted protein. It has the option for the user to select the smiles file of compounds or smiley string. The database gives the parameters such as molecular properties (log P, rotatable bonds, acceptor, donors and surface area), absorption (solubility, intestinal absorption, skin permeability, p-glycoprotein binding), distribution (volume distribution, protein binding, central nervous system and Blood Brain Barrier permeability), metabolism (CYP substrate and inhibitors) and excretion (Total clearance and renal transport). Bisabolene is not reported in pKCSM database; hence, the data are collected from Swiss ADME and TGC system.

Prediction of toxicity

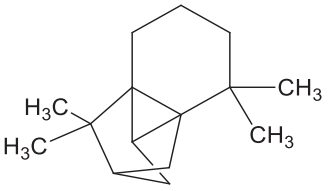
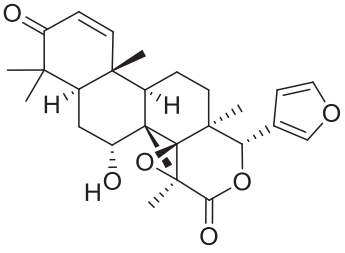
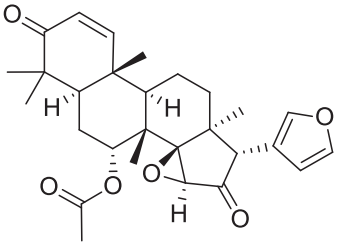
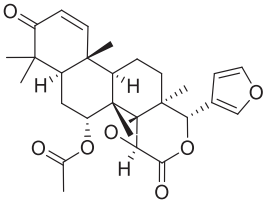
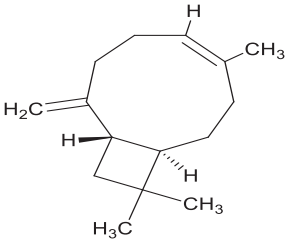
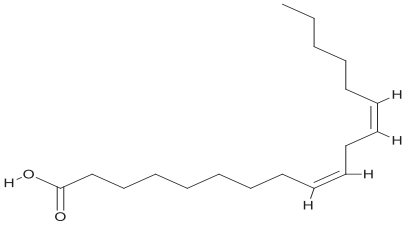
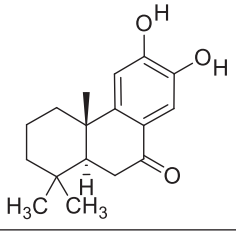
Toxicity studies are important for the molecules to analyse the tolerability toward human and animal use. We used pkCSM online database to determine the toxicity behavior of the 20 selected compounds of neem seeds, which have

Table 1: Structures and reported biological activities of selected neem seed compounds

Name of compound	Structure	Bioactivity
1,2,4 – trithiolane		-
1-Napthalenol		Anti-depressant (Vukics *et al*., 2002), Antiprotozoan activity (Roy *et al*., 2013).
Arachidic acid		Antibacterial and antifungal (Agoramoorthy *et al*., 2007)
Azadirachtin D		Antifeedant activity[52] (Sharma *et al.* 2003)
Azadirachtin H		Anti-feedant activity, anti-fungal (Sharma *et al*., 2003)
Azadiradione		Antimycobacterial, anticancer (Maneerat *et al*., 2008)
Azadirone		Anticancer activity (Gupta *et al*., 2013)
Bisabolene		Anti-viral (Gavanji *et al*., 2015)

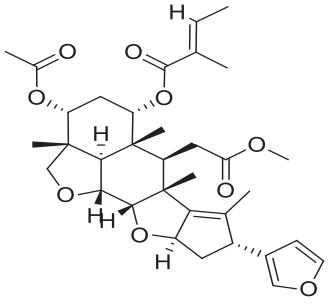
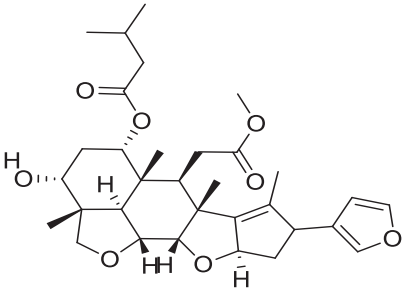
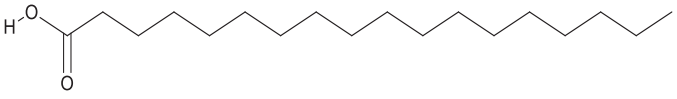
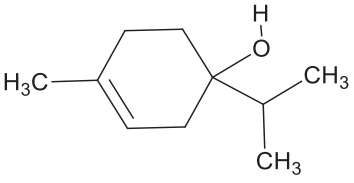
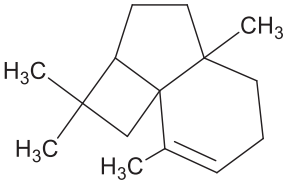
(Contd...)

Table 1: *(Continued)*

Name of compound	Structure	Bioactivity
Cycloisolongifolene		Antiviral activity[32] (Parida *et al.* 2022) Anti-inflammatory[33] (Naik *et al.* 2014)
Desacetylgedunin		Larvicidal,[34] Anti-inflammatory[35] (Sarigaputi *et al.* 2015)
Epoxyazadiradione		Anticancer, antimalarial[29] (Nathan *et al*. 2005)
Gedunin		Antiviral (Dengue virus)[36] (Amraiz *et al.* 2017)
Isocaryophyllene		Anticancer[37] Antiviral[38] (Gyrdymova *et al.* 2019, Legault *et al.* 2007)
Linoleic acid		Antiviral[39] (Bassaganya *et al.* 2003)
Nimbidiol		Anti-diabetic[40] (Mukherjee *et al.* 2013)

(Contd...)

Table 1: *(Continued)*

Name of compound	Structure	Bioactivity
Salannin		Anti-viral (Zika virus)[41] (Koul *et al*. 2004, Priya *et al.* 2018)
Salannol		Anti-feedant[42] (Ezzat *et al.* 2018)
Stearic acid		Antiviral[43] (Tiwari *et al.* 2010)
Terpinen-4-ol		Anti-inflammatory[44] (Hart *et al.* 2000)
α- Panasinsen		Anti-microbial, Anti-plasmodial[45] (Kozykeyeva *et al*. 2020)

shown good binding energy toward COVID-19 protein. The molecules were selected as SMILES format or SMILES string. The software provides the AMES toxicity, tolerated dose, hERGI and II inhibitor, LD50, LOAEL, Skin sensitisation, hepato-toxicity, T.pyriformis, and minnow toxicity (Pires *et al*. 2015).[47]

Molecular dynamics (MD) simulations of ligand and protein structure and MM-PBSA analysis

MD simulation was performed with the minimum energy docked structure using GROningen Machine for Chemical Simulation (GROMACS) (Version 5.1.2) (Kumari *et al.* 2014) [55] with CHARMM36-march2019[56] force field using TIP3P model (Boonstra *et al.* 2016).[57] CHARMM General Force Field server (https://cgenff.umaryland.edu/) is used to prepare the necessary files of ligands. PBC was applied by generating a dodecahedronbox. All the systems were neutralised by adding adequate number of Na^+ or Cl^- ions. After energy minimization, the system was equilibrated for 100ps at 300K using isochoric-isothermal (NVT) equilibration keeping the 2fs time step. The isothermal-isobaric (NPT) ensemble was then performed for 100ps at 300K with the same time step. Electrostatic and van der Waals interactions cutoffs for both NVT and NPT were kept at 1.2 nm. Smooth particle mesh Ewald method was applied for the calculation of long-range interactions (Liu *et al.* 2005, Paliwal *et al.* 2013).[58] Finally, 15000ps MD-simulation was performed using the same cutoff. Further 15000ps trajectories were submitted to MM-PBSA analysis with 20000 frames for human ACE2, RdRp protein, main proteases, and 5000 frames for the spike protein using MMPBSA GROMACS tools (Berendsen *et al.* 1995).[62,63]

RESULTS AND DISCUSSION

Molecular docking

We have selected 20 compounds from neem seed previously reported as antimicrobial or anticancer for docking against main protease, ACE2 receptor, spike protein, and RdRp. Table 2 illustrates the binding affinity (kcal/mol) for the said proteins. The binding affinity ranges from −2.4 to −7.9 for M^{pro}, from −2.4 to −9.2 for ACE2, from −2.5 to −10.0 for spike protein, and from −2.5 to −7.5 for RdRp. We found a highest binding affinity of −7.9 kcal/mol for desacetylgedunin

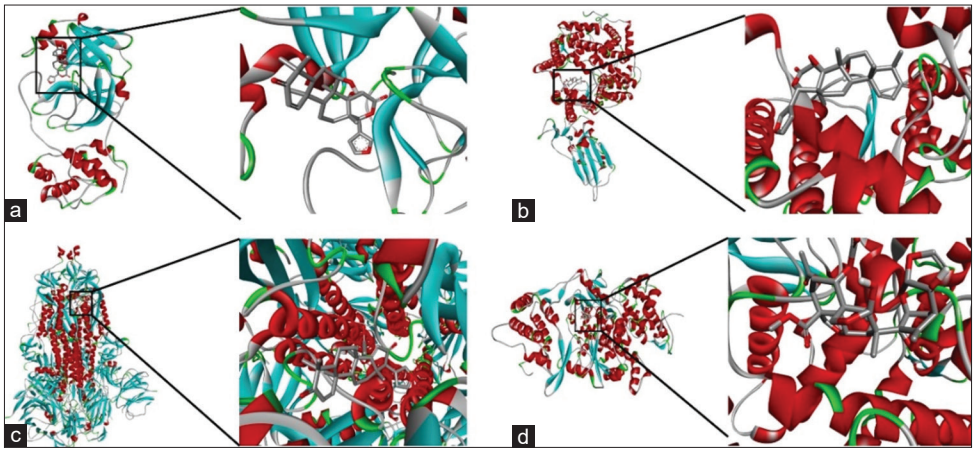


Figure 1: Docked conformation of Desacetylgedunin with (a) Main protease of severe acute respiratory syndrome coronavirus II (SARS-CoV-II), (b) angiotensin-converting enzyme II, (c) Azadiradione with spike protein of SARS-CoV-II, (d) Azadirachtin H with RNA dependent RNA polymerase of SARS-CoV-II

Table 2: Docking scores of compounds obtained from neem seed and reference drug against SARS-CoV-II

Name of compound	Mol. weight (g/mol)	PubChem CID	Docking score (Kcal/mol)			
			Main protease	ACE2	SPIKE	RdRp
1,2,4 – trithiolane	124.3	9258	−2.4	−2.4	−2.5	−2.5
1−Napthalenol	144.17	7005	−5.1	−6.1	−6.7	−5.7
Arachidic acid	312.5	10467	−4.6	−5.5	−5.6	−3.7
Azadirachtin D	676.7	6443232	−5.9	−7.8	−8.0	−7.1
Azadirachtin H	662.7	16722121	−7.7	−8.3	−9.2	−7.5
Azadiradione	450.6	12308714	−7.8	−8.2	−10.0	−7.3
Azadirone	436.6	10906239	−7.8	−8.1	−8.2	−7.4
Bisabolene	204.35	3033866	−5.7	−6.4	−6.7	−6.2
Cycloisolongifolene	204.35	563197	−5.0	−5.8	−6.6	−5.2
Desacetylgedunin	440.5	3034112	−7.9	−9.2	−9.2	−7.3
Epoxyazadiradione	466.6	49863985	−7.5	−8.4	−9.0	−7.2
Gedunin	482.6	12004512	−7.0	−9.1	−8.6	−7.4
Isocaryophyllene	204.35	5281522	−5.2	−6.6	−7.0	−5.6
Linoleic acid	280.4	5280450	−5.0	−5.9	−6.2	−4.4
Nimbidiol	274.35	11334829	−7.1	−7.5	−8.7	−7.4
Salannin	596.7	6437066	−6.5	−7.0	−8.3	−7.0
Salannol	556.7	157144	−6.8	−7.0	−8.5	−6.8
stearic acid	284.5	5281	−4.3	−5.4	−5.8	−4.2
Terpinen-4-ol	154.25	11230	−4.7	−5.5	−5.7	−5.2
α- Panasinsen	204.35	578929	−4.9	−6.1	−7.3	−5.4
Hydroxychloroquine	335.9	178396	−6.3	−6.8	−6.5	−5.6
Remdesivir	602.6	121304016	−7.9	−7.3	−7.7	−7.6
Lopinavir	628.8	92727	−7.9	−7.9	−9.6	−8.3

with M^{pro}, −9.2 kcal/mol for desacetylgedunin with ACE2 receptor, −10.0 kcal/mol for azadiradione with spike protein, and −7.5 kcal/mol for azadirachtin H with RdRp. The binding mode along with the nearby residue with the selected compound having highest affinity is plotted and shown in the Figure 1. It is clear from the plot that there are three types of attractive forces (H-bonding, electrostatic, van der Waals) are operating between the target and receptor molecule. The docked structure showed desacetyl gedunin formed different non-covalent interactions such as electrostatic interactions with the amino acids CYS^{145}, SER^{144}, GLY^{143}, LEU^{141}, HIS^{41} $ASN^{142,}$ and Van der Waals interactions to MET^{165}, GLN^{189}, MET^{49}, and Thr^{24}at the active sites of SARS-CoV-2 main protease (PDB ID-6LU7). In case of ACE II, desacetyl gedunin formed electrostatic interactions with the amino acids GLN^{81}, GLN^{101}, ASN^{103}, GLN^{102}, ASN^{194}, HIS^{195}, GLN^{98}, $ASN^{210,}$ and van der Waals interactions to LEU^{85} and GLU^{208} at the active sites of ACE II (PDB ID-6LZG). Against SARS-CoV-II SPIKE prote in the docked structure showed azadiradione formed electrostatic interactions with the amino acids TYR^{904}, GLY^{908}, ARG^{1107}, GLY^{910}, LYS^{1038}, ILE^{909}, GLY^{908}, TYR^{1047}, $ALA^{890,}$ and van der Waals interactions to ASN^{907}, GLN^{1036}, TRP^{886}, and VAL^{1040}at the active sites of SARS-CoV-2 SPIKE protein (PDB ID- 6VXX). Azadirachtin showed highest docking score against SARS-CoV-2 RdRp and formed electrostatic interactions with the amino acids SER^{682}, ARG^{624}, THR^{680}, ASP^{623}, ASN^{691}, ASP^{760}, ARG^{553}, $ARG^{555,}$ and Van der Waals interactions to SER^{681}, CYS^{622}, THR^{556}, and ASP^{452}at the active sites of SARS-CoV-IIRdRp (PDB ID-6M71). The lowest H-bond distance between H-atom of hydroxyl group present in Azadirachtin H and ARG^{553} is 2.1Å. The nearest residues after docking as illustrated above are given in Table 3.

The docked conformation of neem seed compounds which shows highest binding affinity with the respective protein is shown in Figure 1.

A 3D plot indicating the donor and acceptor region of H-bond interaction for individuals and a 2D contour plot at the active binding sites is shown in Figure 2.

Another descriptor of binding affinity of neem compounds with the protein is studied by Ramachandran Plot shown in Figure 3. In the entire plot, it is seen that in all the docked structures maximum residues are lying in the parallel beta sheet and there are some number of alpha sheets which are also parallel and more flexible.

Molecular docking study was done to see the type of interactions between the natural compounds and the target molecule. Out of total 20 compounds 12 compounds against Spike protein, 10 compounds against ACE 2, 10 compounds against RdRp, 10 compounds against SARS-CoV-2 main protease, and total 11 compounds against all the targets showed good docking score and are tabulated in Table 2. The docking results are quite satisfactory compared to the docking results of other FDA approved drugs such as lopinavir, remdesivir, or hydroxychloroquine [Table 2].

Prediction of ADME

The ADME prediction has been done using pkCSM online database. The molecular properties of the molecules such as molecular weight, logP, hydrogen acceptor, donor, and surface are mentioned in supplementary data. The pharmacokinetic properties analysis showed all of the compounds were absorbed from the gastro-intestinal part, showed less blood brain barrier permeability, and did not affect cytochrome CYP2D6, CYP2C9, and CYP2D6. The skin permeability ($logK_p$) of all selected compounds has been found between 2.7 and −3.28. None of the compounds have affected the renal OCT2 substrate and renal clearance was found to be in between 0.066 and 4.97. All the ADME parameters of the selected compounds have been mentioned in the supplementary data.

Prediction of toxicity

The prediction of toxicity of neem seed compounds was done using pkCSM online database. All compounds were screened for the toxicity study, none of the compounds showed AMES toxicity, hERGI inhibition activity. The LD50 values of all compounds were found between 2.0 and 3.7 (mol/kg), and the chronic oral rat toxicity

Table 3: Interacting/neighboring residues of protein binding with neem seed compounds

Protein	Drug	Neighbouring residues after docking
M^{pro}	Desacetylgedunin	MET A165, GLU A166, GLN A189, CYS A145, SER A144, GLY A143, LEU A141, MET A49, HIS A41, ASN A142, THR A24
ACE II	Desacetylgedunin	GLN A81, GLN A101, LEU A85, ASN A103, GLN A102, ASN A194, HIS A195, GLN A98, ASN A210, GLU A208
Spike	Azadiradione	ASN A907, TYR A904, GLY A 908, ARG A1107, GLN A1036, TRP A886, GLY C910, LYS C1038, ILE C909, GLY C908, VAL C1040, TYR C1047, ALA A890
RdRp	Azadirachtin H	SER A 682, SER A681, ARG A624, THR A680, ASP A623, ASN A691, CYS A622, ASP A760, ARG A553, ARG A555, THR A556, ASP A 452

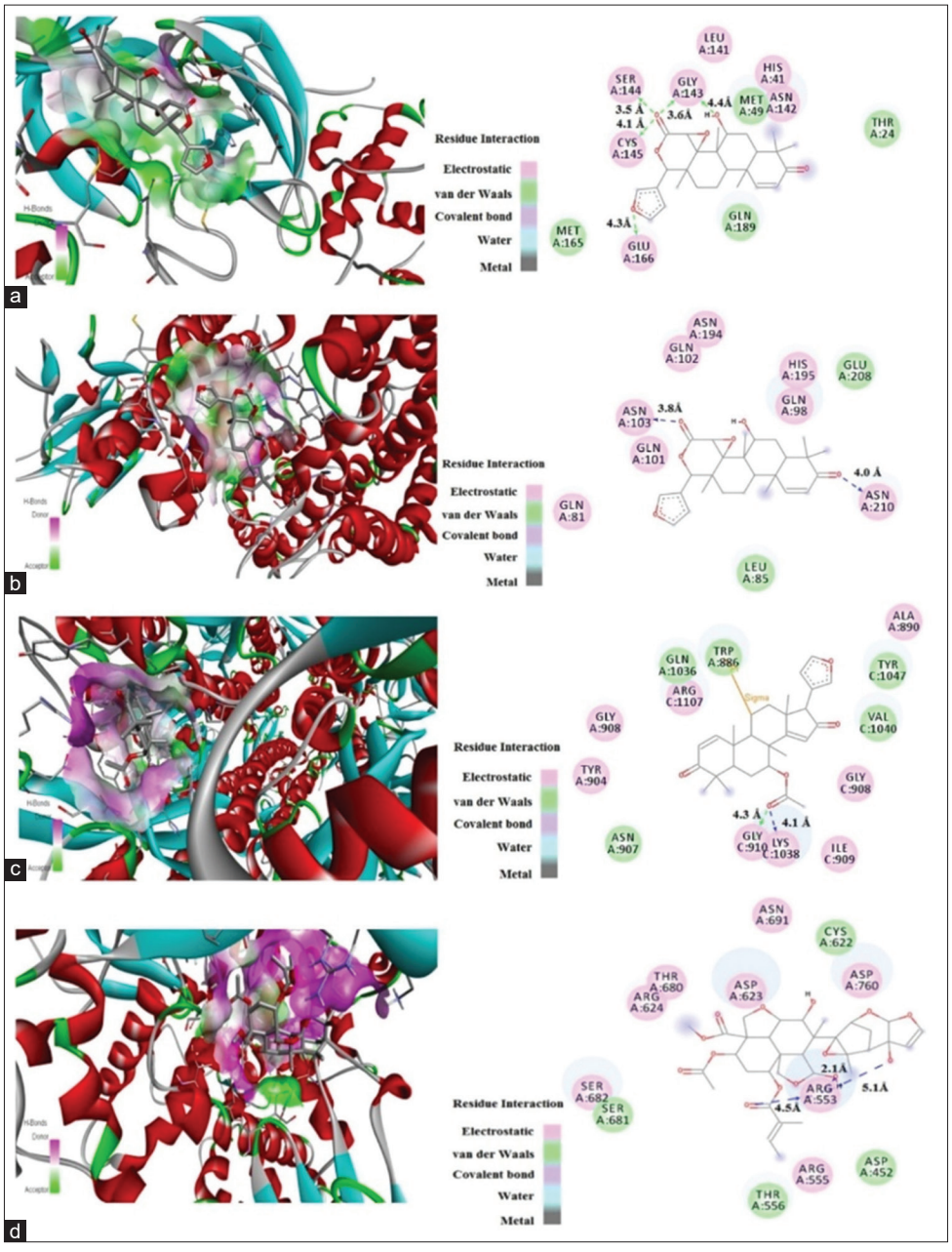


Figure 2: 3D structure of protein-ligand interaction on left and 2D animated pose showing non-covalent interactions between Desacetylgedunin and (a) Main protease of severe acute respiratory syndrome coronavirus II (SARS-CoV-II), (b) angiotensin-converting enzyme II, (c) Azadiradione and SPIKE protein of SARS-CoV-II, (d) Azadirachtin H and RNA dependent RNA polymerase of SARS-CoV-II

(LOAEL) values were found to be between 0.8 and 4.0 (log mg/kg_bw/day), these compounds showed no hepatotoxicity except salannol. All the selected compounds did not show any skin sensitivity. The toxicity value for T. pyriformis and minnow is mentioned in Table 4.

The highest docking scores showed by desacetylgedunin against the main protease and ACE2 protein can be correlated with previously reported anti-inflammatory and antimalarial activity. The docking score of desacetylgedunin was similar to FDA approved protease blocker lopinavir. Two compounds from neem seeds, azadiradione and azadirachtin H showed good binding toward SARS CoV II spike and RdRp protein, respectively, which is quite similar to that of FDA approved RdRp blocker remdesivir (Docking score: 7.6Kcal/mol). Azadirachtin is a well-known compound in agriculture industry because of its pesticidal activity.[28,48,49] Azadirachtin acts mainly as a growth disruptor. Azadiradione possess antimicrobial and anticancer property.[29] Similarly, Desacetylgedunin, Azadirachtins, Azadiradione other limonoids such as Azadirone, Bisabolene, Desacetylgedunin, Epoxyazadiradione, Gedunin, Nimbidiol, Salannin also showed good docking score against the target proteins

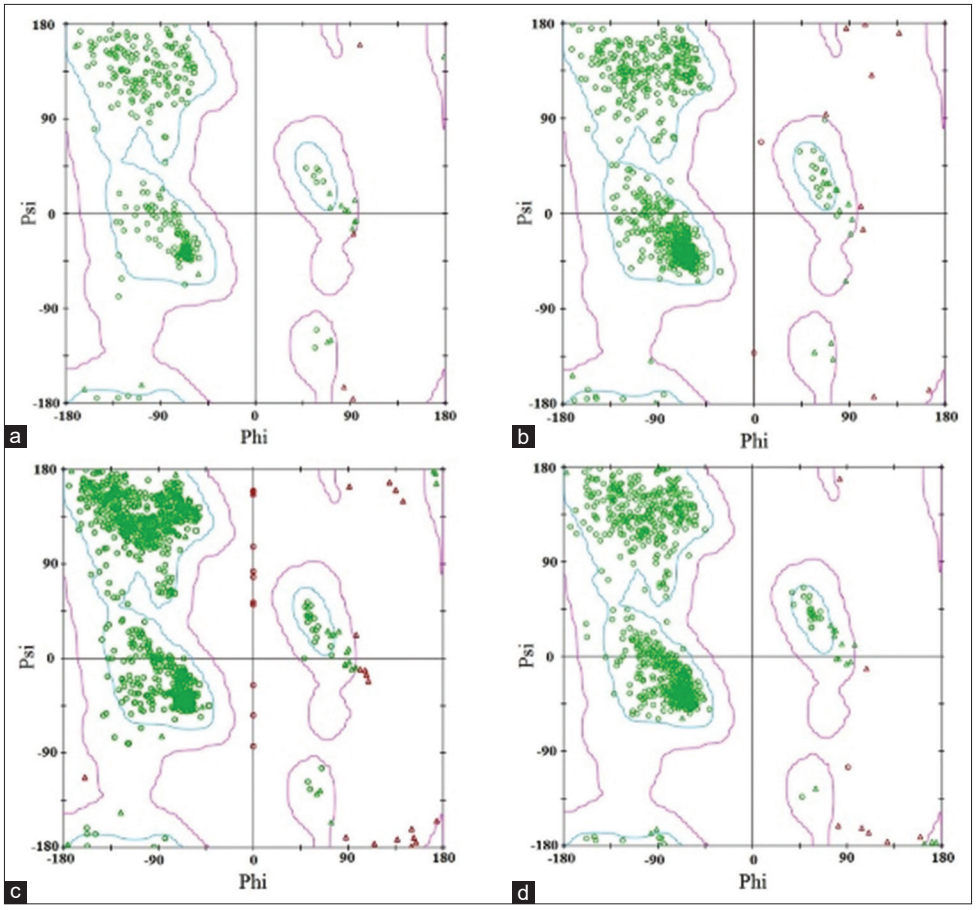


Figure 3: Ramachandran plot of (a) desacetylgedunin and severe acute respiratory syndrome coronavirus II (SARS-CoV-II) Main protease, (b) desacetylgedunin and angiotensin-converting enzyme II, (c) azadiradione and SPIKE SARS-CoV-II protein, (d) azadirachtin H and SARS-CoV-II RNA dependent RNA polymerase Complexes

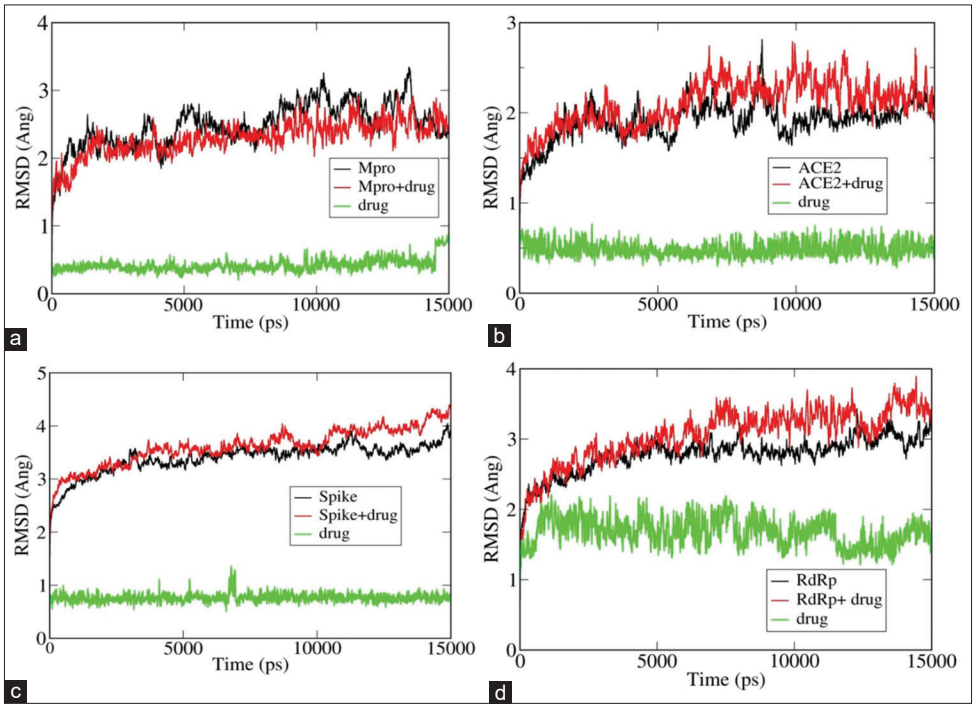


Figure 4: Root mean square division plot of (a) Mpro (b) angiotensin-converting enzyme2 (c) Spike and (d) RNA dependent RNA polymerase in docked and undocked structures

Table 4: Toxicity prediction of neem seed compounds

Compound	AMES toxicity	Max. tolerated dose (human)	hERG I inhibitor	hERG II inhibitor	Oral Rat Acute Toxicity (LD50) (mol/kg)	Oral Rat Chronic Toxicity (LOAEL) (log mg/kg_bw/day)	Hepatotoxi -city	Skin Sensitisation	T.Pyriformis toxicity (log ug/L)	Minnow toxicity (log mM)
Azadirachtin H	No	−1.12	No	No	3.71	2.858	No	No	0.285	9.192
Azadirachtin D	No	−1.098	No	No	3.698	2.919	No	No	0.285	9.456
Azadiradione	No	−0.278	No	Yes	2.037	1.048	No	No	0.726	−0.914
Azadirone	No	−0.285	No	Yes	2.143	1.548	No	No	0.749	−1.296
Desacetylgedunin	No	−0.73	No	No	2.581	0.872	No	No	0.328	0.456
Epoxyazadiradione	No	−0.558	No	No	2.221	0.872	No	No	0.379	−0.021
Gedunin	No	−0.544	No	No	2.55	0.906	No	No	0.329	0.65
Nimbidiol	No	−0.182	No	No	2.304	1.732	No	No	1.461	0.085
Salannin	No	−0.308	No	No	2.234	0.998	No	No	0.287	0.203
Salannol	No	−0.556	No	No	2.187	1.146	Yes	No	0.289	−0.66

which supports their growth regulating activity on insects as well as antiviral (HIV), antibacterial, anticancer, antimalarial, antifungal and many other pharmacological activities like cardioprotective activity on humans.[28,50] To verify the cytotoxicity of the selected compound, we also analysed the ADME cytotoxicity, hepatotoxicity and minnow toxicity by pkCSM online database.[47] The properties of these compounds confirmed that they have permeability through the specific membrane, can distribute in the system and binds with the specific proteins. The ADME analysis also showed that the selected molecules can be well absorbed from the gastro-intestinal tract. The pharmacokinetic parameter depends on the absorption of the molecules which depends upon the lipophilicity and solubility. These compounds of neem seeds contain both the lipophilic and hydrophilic properties, some appeared to be highly lipophilic and some of them appeared to be highly hydrophilic. Both properties are required for the absorption and solubility of the molecules in water. The analysis showed that the compounds are able to release the phosphate group from the ATP and leads to binding of this to glycoprotein, hence most of the compounds having p-gp substrate activity. These compounds do not interfere with the liver metabolism by blocking the CYP1A2 cytochrome enzyme; hence, metabolism of various therapeutic drugs such as anti-malarial, anti-convulsant, and anti-cancer drugs will remain unaffected. These compounds do not block the metabolism of antihypertensive, anti-depressant which are metabolized by CYP2D9. NSAIDS are metabolized by the CYP2C9; these molecules also have no effect on this enzyme. Thus, these compounds have less or no involvement in drug–drug interaction. The toxicity study also predicted that these compounds are safe and may be given as a drug with value for tolerance as human and animal use.

Furthermore, we have performed MD simulation with the docked structure to study the effect of the drugs on the respective protease, RNA, and enzyme of SARS-CoV-2. 15000ps MD results showed that all the drugs have a huge impact on the molecular structure of Mpro, ACE2, Spike, and RdRp. Figure 4a-d showed the root mean square division (RMSD) plots of the undocked and docked structure of the protease, RNA and enzyme. It is clear from Figure 4a that the drug desacetylgedunin stabilize Mpro with time. The undocked Mpro is fluctuated more throughout the simulation whereas the desacetylgedunin docked Mpro gets stabilized after 2000ps. Again, in case of ACE2 the undocked form realises more fluctuation while the desacetylgedunin docked becomes stabilized after 7500ps. Azadiradione stabilizes spike protein which is revealed from the comparatively lower fluctuation of the docked structure of spike. Again, azadirachtin HdestabilizeACE2 receptor strongly after 13000ps which is clear from the RMSD plot as shown in Figure 4d. Figure 5a-d illustrates the root mean square fluctuation (RMSF) of the undocked and docked structure of the protease, RNA, and enzyme.

It is clear from the RMSF plots there is a huge structural fluctuation in the residues during the MD-simulation. Results analyzed by RMSD supports from the RMSF plot.

Radius of gyration (Rg) indicates the compactness of the structure. Generally, system having higher value of Rg represents the higher compactness of the structure. Figure 6a-d represents the radius of gyration plot for undocked and docked Mpro, ACE2, Spike, and RdRp. From Figure 6a, it is clear that the docked structure is more compact with respect to undocked one which is also supported from RMSD plot for Mpro. We found the same scenario for ACE2. From Figure 6b the docked structure is more compact after 4000ps while the undocked one

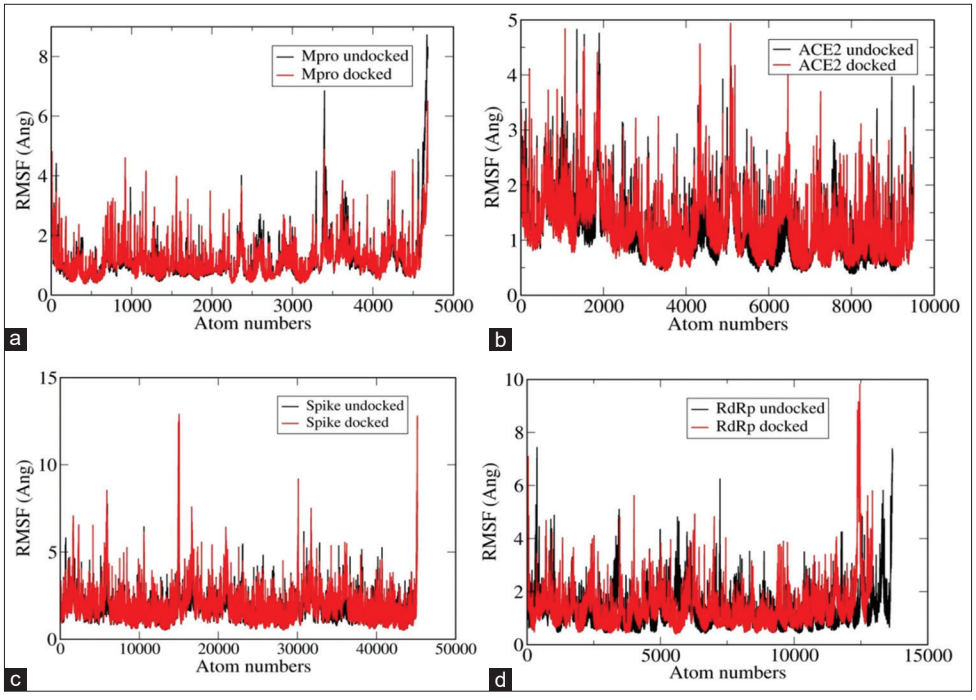


Figure 5: Root mean square fluctuation plot of (a) Mpro (b) angiotensin-converting enzyme2 (c) Spike and (d) RNA dependent RNA polymerase in docked and undocked structures

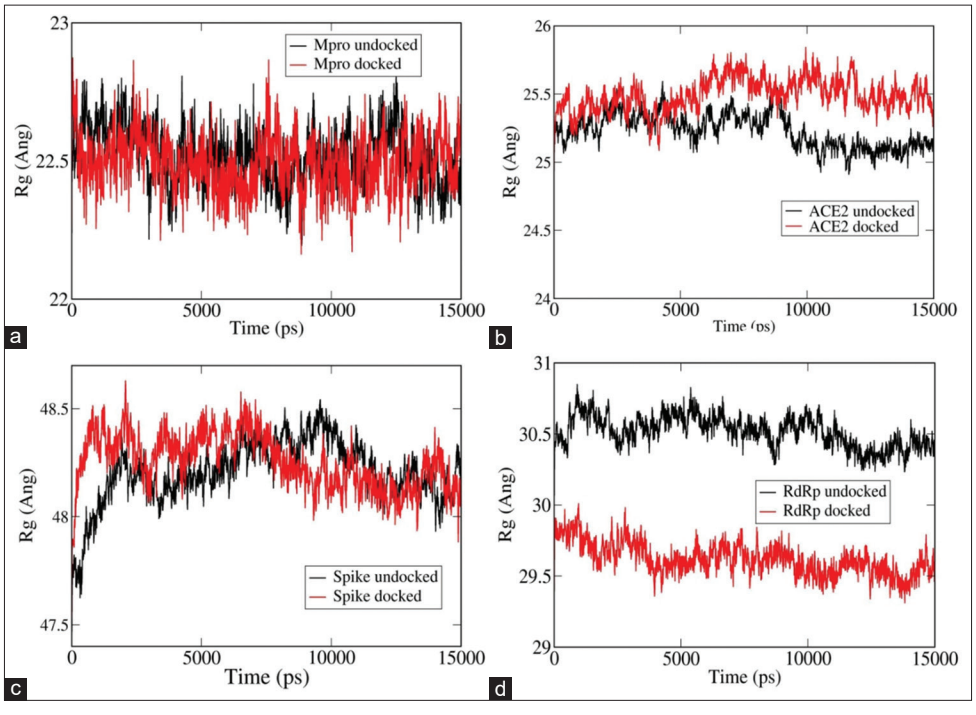


Figure 6: Radius of gyration of (a) Mpro (b) angiotensin-converting enzyme2 (c) Spike and (d) RNA dependent RNA polymerase in docked and undocked structures

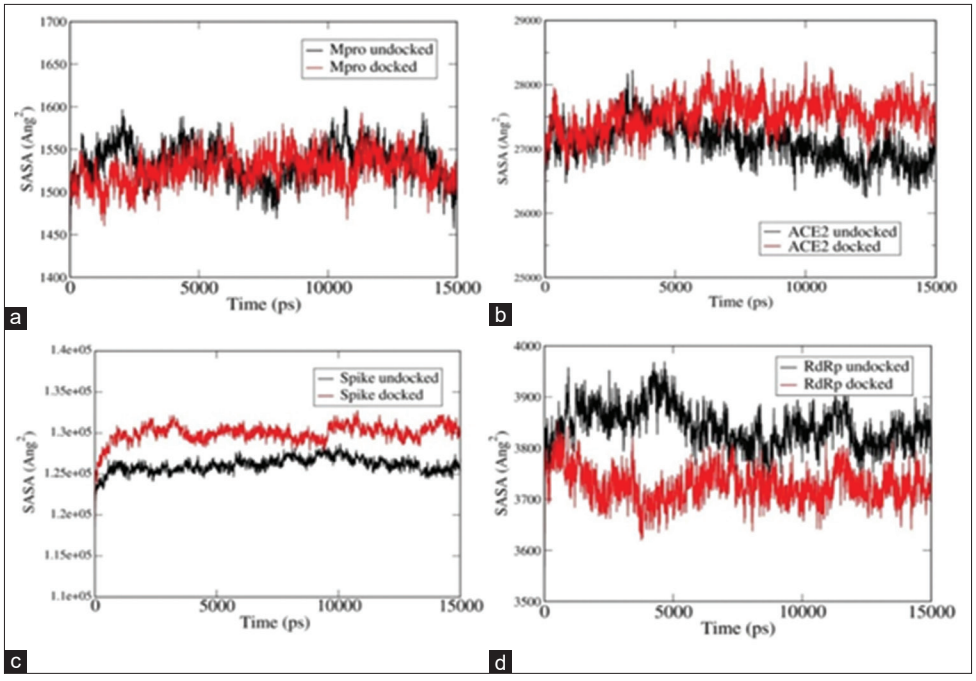


Figure 7: Solvent Accessible Surface Area plot (a) Mpro (b) angiotensin-converting enzyme2 (c) Spike and (d) RNA dependent RNA polymerase in docked and undocked structures

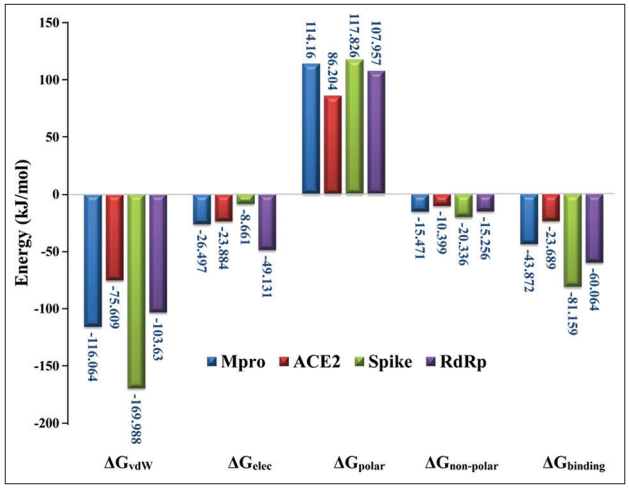


Figure 8: Thermodynamic parameters of the protein-ligand complexes

becomes loss its compactness with time and these results are in concordance with the RMSD results of ACE2. Figure 6c indicates the compact structure of the docked structure which showed similarresultswith the RMSD plot. RdRp becomes desstabilized after docking revealed from Figure 6d which is in accordance with RMSD plot. Figure 8a-d represents the Solvent Accessible Surface Area (SASA) plot. SASA plot of docked Mpro with more surface area indicates its stability during the simulation. Figure 7b and c revealed that docked form of the proteins are more stable due to more solvent accessible surface area which makes the docked structure more stable compare to undocked one. From SASA plot of RdRp, it is clear that it becomes destabilize after docking. Hence, all these results obtained from the MD-simulation are in concordance with each other.

As the target molecules are much more flexible with large sizes, the contribution of binding free energy term to the conformational changes cannot be ignored. To understand the stability of the protein-ligand complex, we have analyzed binding free energies of the composite system as shown in the Figure 8. We found the highest free energy value for weak interaction (vander waal) for spike-azadiradione composite and lowest for ACE2-desacetylgedunin complex. Similar trend has been obtained for the binding free energy of these complexes. The free energy for electrostatic interaction corresponds highest value for RdRp-azadirachtin H complex while the lowest one assigned to spike-azadiradione complex.

CONCLUSION

Our study based on molecular docking revealed very satisfactory result as around 10 out of 6 ADME analysis. MD simulation study of the docked structure of Mpro, ACE2, Spike protein, and RdRp showed that these drugs have the huge impact on these structures which are confirmed by RMSD, RMSF analysis, radius of gyration analysis, and SASA plot analysis. MM-PBSA analysis confirmed that spike-azadiradione composite has the best binding, polar, non-polar, and van der walls free energies and lowest electrostatic interaction, whereas ACE2-desacetylgedunin complex showed all the lowest free energies except the electrostatic interaction. Hence, it can be concluded that these compounds can create a new

way toward the development of new safe drugs based on natural compounds against SARS-CoV-II with no side effects. These compounds can be analysed further to find significant inhibitors of SARS-CoV-II replication and also to find their efficacy to prevent the entry of the virus into the host cell by further biological as well as pharmacological point of view.

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DISCLOSURE STATEMENT

We declare that we have no conflict of interest to disclose.

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