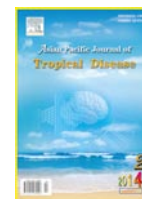




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In silico analysis and identification of novel inhibitor for new H1N1 swine influenza virus

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ABSTRACT

Objective: To identify alternative drug for the treatment of pandemic disease caused by influenza virus.

Methods: The structure based drug design approach was employed. New sequence was employed to build the N1 simulation structure by homology modeling which was further checked for high reliability by verify score and Ramachandran plot. Evaluation of drug likeness and absorption, distribution, metabolism, excretion, toxicity showed that the ligands satisfy all the properties to be used as a drug. Docking studies were performed using LeadIT and docking scores indicated good binding energy values towards N1.

Results: Four candidates were screened and suggested as potent target candidates from the docking studies. The screened compounds from Stemonaceae family illustrated better activity compared to the drugs which are already present in the market.

Conclusions: The results may help to find the alternative drug to solve the drug-resistant problem and stimulate designing more effective drugs against 2009–H1N1 influenza pandemic, yet pharmacological studies have to confirm it.

1. Introduction

Global outbreak of flu caused by a new strain of influenza A virus subtype H1N1, commonly referred to as swine flu identified in April 2009 which was infected and transmitted between humans[1]. It is thought to be a mutation, more specifically a reassortment, of four known strains of influenza A virus. The U.S. Center for Disease Control and Prevention warned that the outbreak could be pandemic. On April 2009, the World Health Organization raised their alertness level from 3 to 4 worldwide in response to sustain human-to-human transfer of the virus, and the situation was raised to level 5 on 29 April 2009. Furthermore, on June 11, 2009, the World Health Organization declared an H1N1 pandemic, moving the alert level to phase 6, marking the first global pandemic since 1968. Hence, there is an urgent need to find the resolution for this international problem. Unfortunately, H1N1 virus was reported that it has gained drug resistance for oseltamivir[2]. Hence, a new drug is required against this

epidemic disease.

Swine influenza A virus belong to the viral family of Orthomyxoviridae. They are RNA viruses with a segmented genome that is comprised of eight negative-sense, single-stranded RNA segments. These eight segments encode eleven proteins in which two are surface glycoproteins[3], hemagglutinin (HA) and neuraminidase (NA). HA has 16 subtypes (H1, H2, H3, ..., H16) and NA has 9 subtypes (N1, N2, N3, ..., N9) and this novel virus consists of subtype H1 and N1[4]. HA binds with sialic acid located on the surface of the targeted host cell to initiate virus infection and sialic acid was removed from virus by NA[5]. By the above two steps process, HA and NA improve virus releasing and the spread of infection to new cells, respectively[6]. By blocking HA or NA could prevent virus from invading into host cells[7]. The Food and Drug Administration (FDA) approved antiviral drugs oseltamivir (Tamiflu) and zanamivir (Relenza) are NA inhibitors[8]. Influenza A virus subtype H1N1 is the most common cause of influenza in humans[9].

Oriental medicinal herbs with antiviral activity are currently in the spotlight as a complementary or alternative medicine. The root of *Stemona tuberosa* Lour is a traditional Chinese medicinal plant known for its antitussive and anti-ectoparasitic activity[10]. Recently, *Stemona tuberosa*

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extracts have been attracting new interest for their multi-biological functions including anti-tuberculous, antifungal, antiviral and anticancer activity^[11]. Alkaloids, stilbenoides, and tocopherols have been identified as main constituents of the plant^[4].

Increasing clinical failures of new drugs call for a more effective use of absorption, distribution, metabolism, excretion (ADME) technologies. Since these technologies are more advanced and reliable in terms of accuracy and predictiveness. An increase in their usage is expected during the initial development and screening phase of innovative drugs. New computational methods including consensus modeling for increase in the accuracy of *in silico* ADME-Tox prediction used for virtual screening in lead optimization^[12].

The objective the study is to figure out potent candidates for N1 for the 2010 outbreak of influenza A H1N1. The latest N1 structure model by homology modeling is built and antiviral compounds from plant of Stemonaceae family are screened by docking study on N1. Candidate ligand poses are evaluated and prioritized according to the binding energy and as well as toxicity analysis.

2. Materials and methods

2.1. Target identification

NA was chosen as drug target. It is a glycoprotein and it has 9 subtypes (N1, N2, N3... N9) which assort the type of influenza A virus. Influenza A virus A/Perth/262/2009 (H1N1) sequence with accession number ADJ67981 was selected for *in silico* analysis.

2.2. Homology modeling and molecular dynamics

The sequences used in the present study appear in National Center of Biotechnology Information with protein accession number ADJ67981. The crystal structure of NA (PDB: 3NSS_A, 3TIA_A) available at Protein Data Bank of The Research Collaboratory for Structural Bioinformatics^[13] was used as a template for constructing the 3-D models of our selected recent N1 sequence. Discovery studio 3.5 was used for homology modeling of protein three-dimensional structures^[14]. The stereochemistry quality of the structures were validated with ProCheck, Whatif and Verify3D. Quality factors for the protein models were calculated using ERRAT2^[15]. Model of NA protein of H1N1 were further processed by applying CHARMM force field. Potential energy of a specified structure was evaluated by using calculate energy protocol of Discovery Studio (DS) 3.5. Energy minimization of 3-D modeled protein structure was done with the help of standard dynamics cascade protocol of DS 3.5 which performs the following steps: minimization with steepest descent method, minimization with conjugate gradient, dynamics with heating, equilibration dynamics, and production dynamics. The minimization protocol minimizes the energy of a structure through geometry optimization. For the simulation cascade, following parameter are used:

steepest descents minimization [500 steps, root mean squared (RMS) gradient 0.1] in first minimization step and in second steepest descents minimization (500 steps, RMS gradient 0.0001), heating (2000 steps, initial temperature 50 K, final temperature 300 K), equilibration (120 ps, 1 fs time step, coordinates saved every 1000 steps) and production (120 ps, 1 fs time step, 300 K, moles, volume and temperature ensemble, non-bond cutoff 14 Å, switching function applied between 10 and 12 Å, coordinates saved every 1000 steps)^[16].

2.3. Binding site prediction

The active site of the protein was predicted using Discovery studio 3.5. It uses receptor cavity method based on eraser algorithm. This study reveals the important residues in the target protein which are responsible for ligand binding, present in the active site or elsewhere^[17].

2.4. Ligand preparation

The list of antiviral compounds from plant of Stemonaceae family was gathered from public domain and the peer published articles. The drug likeliness properties of the selected compounds were investigated with the help of Lipinski drug filter in Discovery studio 3.5^[18]. Library for the screened compounds (structure data format files) were prepared and the energy of the ligand library was minimized using smart minimizer algorithm with the parameters of 200 steps and at RMS gradient 0.1. Each of the minimization methods were carried out with CHARMM force field^[19].

2.5. Absorption, distribution, metabolism, excretion and toxicity (ADMET) investigation

ADMET studies were executed through ADMET descriptors Discovery studio 3.5^[20]. The ADMET studies provides insight into the pharmacokinetic property of all compounds. Toxicity studies includes mutagenicity and carcinogenicity assays. Mutagenicity predicts the ability of the drug to cause mutation to the human cells. Mutagenicity assay is based on the Ames test. Carcinogenicity assay predicts the ability of the compound to cause cancer to normal human cells and carcinogenicity test were carried for mouse and rat models. Toxicity prediction studies serves as a preclinical examination and helps to minimize the time and cost during clinical trials. Toxicity prediction studies were executed through toxicity prediction by computer assisted technology in Discovery studio 3.5^[21].

2.6. Molecular docking

Molecular docking studies were performed to investigate the binding affinities and interaction modes between the inhibitors and the target using BioSolveIT FlexX. The docking score was noted down and docking poses were saved for reference. In a similar manner docking score of two FDA approved commercially available antiviral drugs oseltamivir and zanamivir were also recorded in the docking study for comparison.

3. Results

3.1. Homology modeling and molecular dynamics

The results of alignment of N1 sequences are presented in Figure 1. The sequence identity and similarity of N1 sequences were 36.7% and 50.6%, respectively. Five models were generated and the model showing the least discrete optimized protein energy score (-42708.714844) of the crystal structure of the templates, Protein Data Bank ID (3NNS and 3TIA) was saved for further loop refinement and validation. Loop refinement of the 5 models was done after they are built and energy refinement method gives best conformation to the model. After the Loop refinement the newly built model with all its conserved regions should be refined at the loop regions. Five Loop refinement models were generated and the least discrete optimized protein energy score (-42983.671875) was selected for further study. The similarity between the modeled and the template structure was determined by superimposing of three dimensional structures. Superimpose of modeled and template structure is presented in Figure 2. Reliability of new homology model for N1 was identified by Ramachandran plot and verified score are presented in the Figure 3. The Ramachandran plot indicates the region of possible angle formations by ω (phi) and ψ (psi) angles. The favored and additional allowed regions are 88.2% and 10.3% respectively.

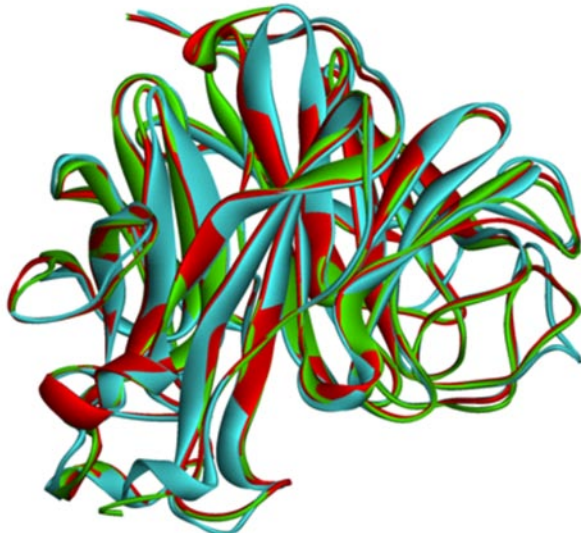


Figure 2. Superimposition of PM0078433 modeled structure. Red: Modeled structure; Blue: 3TIA, chain A; Green: 3NSS, chain A.

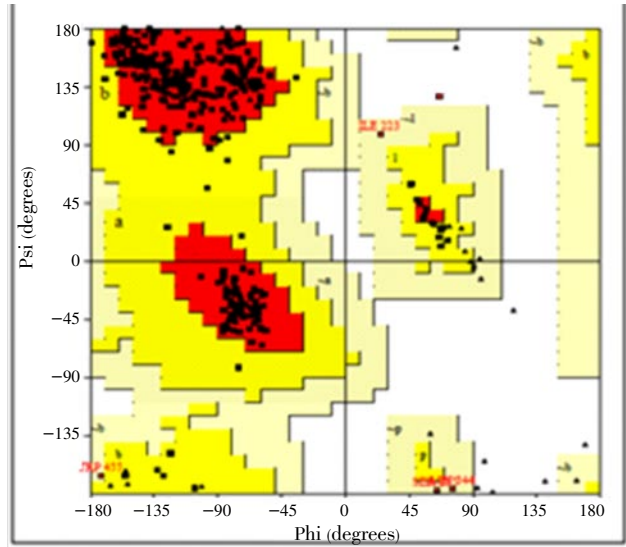


Figure 3. Ramachandran's plot of PM0078433.

Modelled structure was submitted to Protein Model Database which is a repository for three dimensional protein models obtained by structure prediction methods. Submitted NA protein can be downloaded from Protein Model Database using accession number PM0078433. Modeled protein dynamics studies were performed using DS 3.5. The energy minimization studies are performed to calculate the potential energy of the target protein. The potential energy of the target protein should be minimum. The lowest potential energy of the target protein was found to be -21017.20 in 8th conformation. The results of molecular dynamics studies are presented in Table 1.

Table 1

Protein energy minimized data of modeled protein using DS 3.5.

S. No.	Name	Total energy	Potential energy	Temperature (K)	Van der waals energy	Electro statistic energy
1	Conformation	-15 654.60	-20947.10	307.450	-2 309.92	-26 223.80
2	Conformation	-15 653.80	-20904.70	305.038	-2 324.69	-26 288.20
3	Conformation	-15 656.00	-20918.10	305.686	-2 260.08	-26 299.20
4	Conformation	-15 659.60	-20869.10	302.635	-2 262.85	-26 295.50
5	Conformation	-15 661.20	-20911.40	304.996	-2 304.86	-26 275.60
6	Conformation	-15 665.90	-21 000.20	309.881	-2 295.25	-26 344.50
7	Conformation	-15 666.80	-20980.20	308.667	-2 340.62	-26 326.90
8	Conformation	-15 672.70	-21 017.20	310.472	-2 275.73	-26 366.00
9	Conformation	-15 676.70	-20956.90	306.737	-2 268.74	-26 319.30
10	Conformation	-15 680.60	-21 000.60	309.049	-2 285.35	-26 345.90

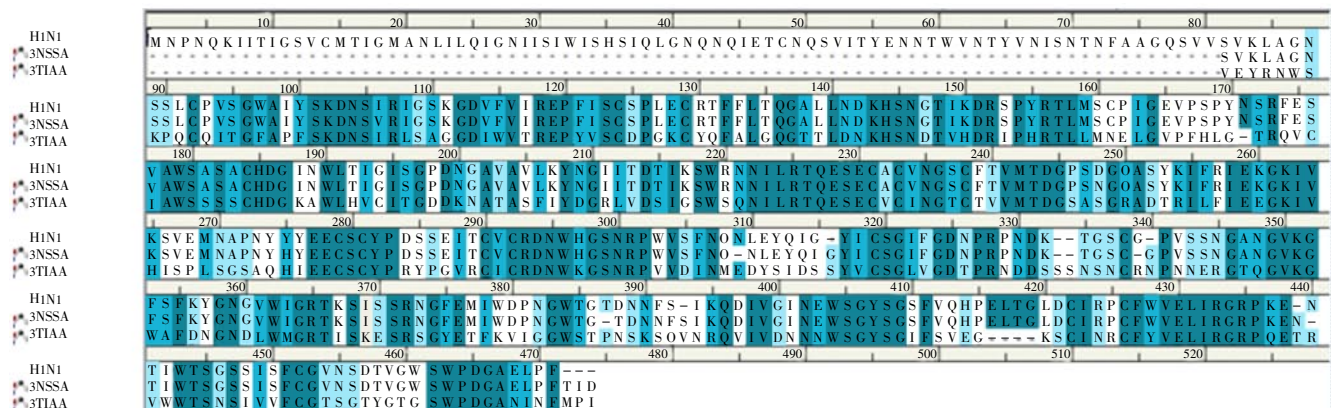


Figure 1. Sequence alignment of ADJ67981 with template sequences. Deep green color: conserved residue in all three sequences.

3.2. Binding site prediction

Based on the receptor cavity method, 17 active sites were identified for the modeled structure PM0078433. Based on size of the volume, the first active site was selected for further study and the details of the active site residues and binding site are; the area of active site 97.75 volume, XYZ coordinates 21.957 A0, 23.072 A0 and 32.747 A0 and 26 amino acids (SER101, ASP103, SER105, ILE117, ARG118, PRO120, PHE121, ILE122, THR131, PHE132, PHE133, CYS161, ILE163, GLY164, GLU165, VAL166, PRO167, TRP423, VAL424, GLU425, LEU426, GLY440, SER441, SER442, ILE443 and SER444) (Figure 4).

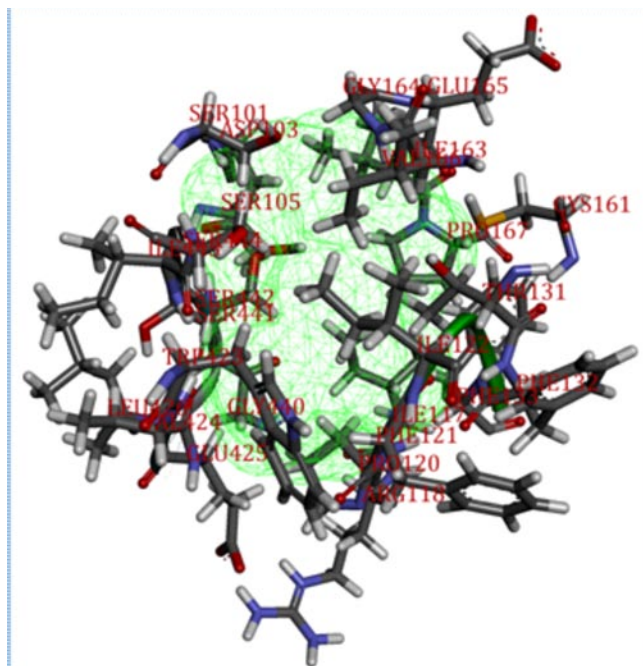


Figure 4. Binding site of modeled protein (PM0078433). Green color indicates binding area of the active site of modeled protein (PM0078433).

Table 2

ADMET properties of the antiviral compounds.

Name	Solubility level	Blood brain barrier level	Extension CYP2D6	Extension hepatotoxic	Absorption level	Extension PPB	AlogP98	Polar surface area 2D
Croomine	2	2	0.019806	-6.03368	0	-6.38	3.063	82.045
Stemonine	2	1	-0.697450	-8.08857	0	-2.35	2.988	29.583
Stilbenoids_19	3	2	-6.817980	-3.58011	0	-7.31	-0.111	76.232
Stilbenoids_23	3	1	-0.800040	-2.95520	0	2.65	4.263	41.631
Tuberospironine	2	1	-1.576760	-6.54295	0	-3.36	3.603	55.814

Solubility: 2–Low soluble, 3–good soluble; Blood brain barrier: 2–medium penetration, 3–Low penetration; CYP2D6: -ve: non-inhibitors, +ve: inhibition; Hepatotoxic: 0–1–Non-toxic; PPB: Greater the value greater the binding capacity; PPB: Plasma protein binding.

Table 3

TOPKAT prediction values of the antiviral compounds.

Name	NTP carcinogenicity call (Male mouse) (v3.2)	NTP carcinogenicity call (Female mouse) (v3.2)	Developmental toxicity potential (v3.1)	Skin irritation (v6.1)	Ames mutagenicity (v3.1)
Stemonine	0.000	0.002	0.002	1.000	0.064
Stilbenoids_19	0.000	0.000	0.006	0.000	0.000
Stilbenoids_23	0.000	1.000	0.000	0.739	1.000
Tuberospironine	0.000	0.002	1.000	0.993	0.998

0: Negative result; 1: Positive result; NTP: National toxicology program.

3.3. Ligand preparation

The set of 108 ligand molecules studied in this work were retrieved from Pub Chem^[22], NCI^[23], Zinc^[24], and Traditional Chinese medicine databases^[25]. Drug likeness determines whether particular molecule is similar to the known drugs. The Lipinski rules of five for the compounds were predicted via Lipinski drug filter. Thirty-one compounds satisfied the Lipinski rule of five. The results demonstrated that the compounds follow Lipinski rule of five and can be strongly recommended as a drug. The energy minimizations of the filtered ligands were performed using DS 3.5. The compound Stilbenoids_19 exhibited minimum potential energy and was found to be -11.398. The results of the energy minimization of the selected ligand molecules are presented in Figure 5.

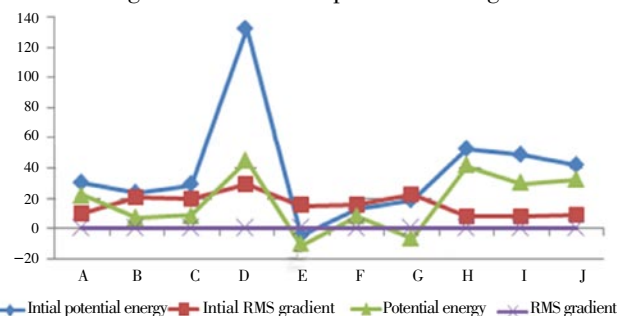


Figure 5. Distribution of ligand energy minimization values.

A: Croomine; B: Stemonine_C; C: Stemonine_D; D: Stemonine; E: Stilbenoids_19; F: Stilbenoids_21; G: Stilbenoids_23; H: Tuberospironine; I: Tuberospironine_H; J: Tuberospironine.

3.4. ADMET properties of antiviral compounds

The ADMET properties of the compounds are depicted in Table 2. Stilbenoids_19 has the solubility level and blood brain barrier of 3 and 2, respectively. The results illustrated that the compounds have good pharmacokinetic properties and it satisfies all the parameters to be taken over as a good drug. The toxicity profiles of the compounds are presented in Table 3.

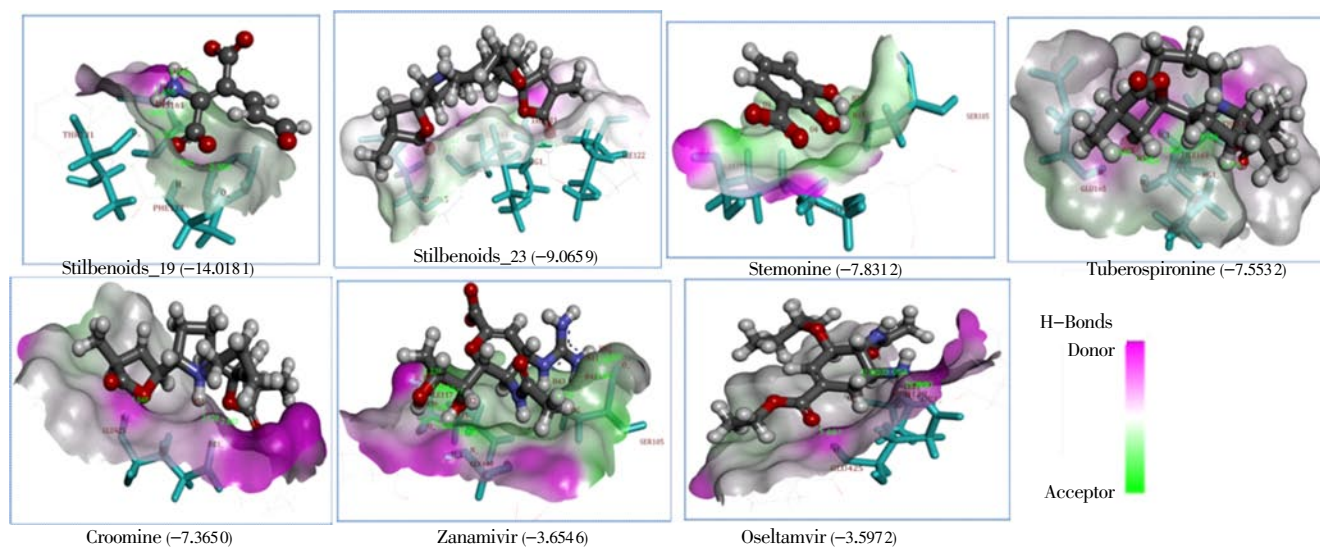


Figure 6. Docking poses of best candidates in N1.

Pink: Donor; Green: Acceptor

3.5. Molecular docking study

The ligand molecules were docked into NA modeled structure. The docking results of the top 5 compounds and FDA approved drugs with NA are presented in Tables 4 and 5, respectively. The docking poses of best compound interaction are revealed in Figure 6. Stilbenoids_19 had the lowest binding energy score of -14.0181 kcal/mol and is even lesser than the standard controls zanamivir that had -3.5972 kcal/mol and oseltamivir and -3.6546 kcal/mol.

Table 4

Docking results of modeled protein N1 with antiviral compounds.

Compound name	Lead-IT (docking)					
	Lead-IT score	H-bond	Amino acid	Amino acid	Ligand atom	H-bond length (nm)
Stilbenoids_19	-14.0181	6	THR131	O_	O2	0.220741
			PHE133	O_	O4	0.236485
			PHE133	H_	O2	0.185986
			CYS161	N_	H15	0.231471
			THR131	O_	H16	0.189968
			CYS161	N_	H16	0.176965
Stilbenoids_23	-9.0659	5	THR131	HG1_	O4	0.201911
			ILE163	O_	O3	0.204388
			ILE163	O_	O5	0.189694
			ILE163	HN_	O2	0.211992
			GLU165	HN_	O5	0.177781
Stemonine	-7.8312	4	ILE117	HN_	O3	0.193543
			GLY440	HN_	O4	0.198523
			SER105	OG_	H15	0.191605
			SER105	OG_	H16	0.161684
Tuberospironine	-7.5532	6	THR131	HG1_	O7	0.211429
			ILE163	O_	O25	0.172223
			ILE163	HN_	O3	0.195641
			GLU165	HN_	O25	0.183567
			ILE163	N_	H51	0.196434
Croomine	-7.365	3	GLU425	OE1_	O1	0.286333
			GLU425	HN_	O2	0.154825
			GLU425	OE1_	H51	0.212776

Table 5

Molecular docking of FDA approved zanamivir and oseltamvir.

Compound name	Lead-IT (docking)					
	Lead-IT score	H-bond	Amino acid	Amino acid	Ligand atom	H-bond length (nm)
Zanamivir	-3.6546	6	SER105	O_	N11	0.231101
			ILE117	O_	O2	0.237100
			GLY440	HN_	O2	0.199522
			ILE117	O_	H35	0.164569
			GLY440	N_	H35	0.222359
			ILE117	O_	H36	0.207947
			SER105	OG_	H41	0.187988
			SER105	O_	H42	0.200653
			SER105	OG_	H43	0.211631
			Oseltamvir	-3.5972	5	PRO120
PRO120	O_	O4				0.290713
GLU425	HN_	O3				0.188715
PRO120	N_	H50				0.235302
GLU425	OE1_	H51				0.185953

Interestingly, remaining compounds were more potent than zanamivir and oseltamvir. The stilbenoids_19 had six hydrogen bond interactions with the modeled protein. The residue THR131 had two hydrogen bond interactions with the bond length of 0.220741 nm and 0.189968 nm respectively, PHE133 had two hydrogen bond interactions with the bond length of 0.236485 nm and 0.185986 nm respectively and CYS161 also had two hydrogen bond interactions with the bond length of 0.231471 nm and 0.176965 nm, respectively.

4. Discussion

The study of molecular docking has emerged during last three decades and is becoming an integral part in drug discovery and development. The study has embarked on to design a new molecular lead by structure based docking procedure using NA as the target. Computer aided drug designing and molecular docking analysis are highly effective in creating and analyzing new candidate drug molecules. The predicted protein homology model had 88.2% and 10.3% favored and additional allowed regions respectively. The

compounds stilbenoids_19, stilbenoids_23, stemonine and tuberospironine were selected as potent target candidate drugs for H1N1. The molecular docking study of N1 with FDA approved drugs zanamivir and oseltamvir was evaluated to identify the similar bioactivity. The docking results showed that stilbenoids_19 had the lowest binding energy of -14.0181 when compared to FDA approved drugs. The docking studies of stilbenoids_19 on the optimized and energy-minimized model of NA showed some important H-bond interactions with functionally important residues. This information can be used for structure-based and pharmacophore-based new drug designing for development of novel therapeutic agents for the prevention and treatment of influenza. A combination of homology modeling, virtual screening and molecular docking, putative novel NA inhibitors can be identified, which can be further evaluated by *in vitro* and *in vivo* biological tests. However, the mechanism is still not clear, further clinical investigations are urgently required, so that the compounds of Stemonaceae can be expected to be a silver lining for latest threat to mankind H1N1 pandemic.

Conflict of interest statement

We declare that we have no conflict of interest.

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