INTRODUCTION

Dengue virus (DENV) is the causative agent of dengue fever which is currently an increasing health problem in many countries. It is a member of the Flavivirus genus of the Flaviviridae family. DENV is transmitted to humans by the bite of *Aedes aegypti* or *Ae. albopictus* female mosquitoes and causes the full spectrum of infection by any one of five serologically related but genetically distinct virus serotypes (DEN 1-5)\(^1\)-\(^7\). In several cases, the disease progresses to serious medical conditions such as dengue hemorrhagic fever\(^8\)-\(^10\) which causes bleeding, low blood platelets count and blood plasma leakage, or dengue shock syndrome defined by a massive increase in the systemic capillary permeability with consequent hypovolemia and dangerously low blood pressure\(^11\)-\(^14\). DENV has a single-stranded RNA genome that is packaged by the virus capsid protein in the host-derived lipid bilayer and surrounded by 180 copies of two glycoproteins. The RNA genome of DENV is about 11000 bases that encode only 10 proteins. Three of these are structural proteins, viz. capsid protein (C), membrane precursor protein (prM) and envelope protein (E), which form the coat of the virus and deliver the RNA to target cells; while seven of them are nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 that coordinate the assembly of new viruses once the virus gets inside the host cell\(^15\)-\(^17\). The non-structural protein NS3 contains a protease (NS3\(_{pro}\)) and a helicase (NS3\(_{hel}\)) domain. The NS3\(_{pro}\) domain mediates the processing of polyprotein in specific site. The NS3\(_{pro}\) domain, the member of S7 family of serine proteases depends upon a cofactor, NS2B to turn into their fully active form. NS2B-NS3\(_{pro}\) of DENV has already been recognized as a potential therapeutic target for the development of new antiviral compounds against DENV\(^18\)-\(^23\).

Therefore, there is a need for screening the new potent inhibitors from various sources using different strat-
egies for the development of effective drugs against DENV. In the area of drug discovery, medicinal plants are one of the most popular sources of effective natural lead compounds. They contain a variety of phytochemicals that can be used as drug against different diseases and infections. There have been numerous reports on the antiviral activity of various plants against dengue viruses\textsuperscript{16, 25}. Neem plant (\textit{Azadirachta indica}) is one among those medicinal plants, which possesses potential antiviral activity against DENV\textsuperscript{26}. More than 135 phytochemicals of diverse structure including triterpenoids have been isolated from various parts of neem plant, but only few of them have been studied for their specific medicinal properties\textsuperscript{27–28}. Triterpenoids are terpenoid derivatives of triterpene molecules which exhibit a wide range of biological properties, including anticancer, antibacterial, antifungal, antimalarial, and antiviral activities. The triterpenoids present in neem like salannin, nimbin, desacetylnimbin, desacetylsalannin, 3-tigloylazadirachtol, azadirachtin, salanninolide, 3-acetyl1-tigloyl azadirachtin, azadirachtin I, azadirachtin D, etc. are known for their specific medicinal activities\textsuperscript{29}. The antiviral activity of triterpenoids from neem plant against DENV NS2B-NS3\textsubscript{pro} has not yet been reported.

In the present study, five triterpenoids from neem plant, namely salannin, nimbin, desacetylnimbin, desacetylsalannin and azadirachtin have been evaluated for their potential inhibitory activity against DENV NS2B-NS3\textsubscript{pro} using \textit{in silico} molecular docking approach. The present study was undertaken to identify an effective and potential drug candidate against dengue infection in view of the fact that till now there is not a single drug available for dengue infection.

\section*{MATERIAL & METHODS}

\subsection*{Receptor and ligands structure}

The crystal structure of complete NS2B-NS3 molecule fused to 18 residues of the NS2B cofactor was retrieved from Protein Data Bank (PDB—Available from \url{http://www.rcsb.org}) using the PDB code: 2VBC. This crystal structure was identified and reported by Luo et al\textsuperscript{30} at a resolution of 3.15 Å. This structure contains two domains: serine protease N-terminal domain and the ATPase/helicase domain located at the C terminus of NS2B-NS3. The serine protease N-terminal domain of NS2B-NS3 was selected for docking studies with small molecules of interest. The 3D structure of triterpenoids of neem plant (azadirachtin, desacetylnimbin, desacetylsalannin, nimbin, and salannin) were searched and downloaded from PubChem database (Available from \url{http://pubchem.ncbi.nlm.nih.gov}) in SDF format. All the visualizations of molecular structure were performed with Pymol\textsuperscript{31} and Ligplot\textsuperscript{32–33} as they are very powerful softwares, for all purpose of molecular visualizations.

\subsection*{Molecular docking}

MTiAutoDock web server\textsuperscript{34} was employed to perform molecular docking between NS2B-NS3\textsubscript{pro} of DENV and selected triterpenoids from neem plant. AutoDock allows to dock compounds into a binding site defined by the user or blind docking using AutoDock 4.2\textsuperscript{35}. The selected ligand molecules and the protein NS2B-NS3 serine protease of DENV were converted in SDF and PDB format respectively, and uploaded into MTiAutoDock server for autonomous docking. The uploaded protein structure in PDB format in MTiAutoDock is automatically cleaned and preprocessed: all hetero atoms (HETATMs) are removed and hydrogen atoms are added to the structure using molecular graphics laboratory (MGL) tools. Further, the calculated active site in the crystal structure of protein was demarcated by using the list of residues (HIS 51, ASP 75 and SER 135) which form a catalytic triad in the binding site of the DENV NS2B-NS3\textsubscript{pro}.

\section*{RESULTS}

\subsection*{Molecular docking}

Molecular docking of small molecules into the binding site of therapeutic target protein is a technique commonly used in the area of drug discovery and molecular interaction studies\textsuperscript{36–38}. The inhibitory activity of small molecules in cell line experiments does not give comprehensive information concerning the binding orientation of ligands with the target protein. But \textit{in silico} molecular docking studies give a straightforward description about the molecular interaction of the compounds with the active site of the protein\textsuperscript{39}. Hence, docking analysis was performed to predict the inhibitory potential of selected neem triterpenoids against DENV NS2B-NS3\textsubscript{pro} and also to determine the amino-acid residues of DENV NS2B-NS3\textsubscript{pro} involved in interaction with triterpenoids. Five chemically diverse neem triterpenoids including azadirachtin, desacetylnimbin, desacetylsalannin, nimbin and salannin (Figs. 1 a–e) were selected to study their effect on dengue NS2B-NS3\textsubscript{pro}. These small molecules were docked into the receptor structure to form complexes. Docking generated several structures and those having high docking scores were selected for further studies. The molecular docking results showed that nimbin, desacetylnimbin and desacetylsalannin have good binding affinities with DENV NS2B-NS3\textsubscript{pro}, while
azadirachtin and salannin did not show any interaction with the target protein. Our finding substantiates the report of Parida et al\textsuperscript{26}, in which they have reported that azadirachtin is not capable of inhibiting the replication of dengue type-2 through their \textit{in vitro} and \textit{in vivo} studies. It was observed that the DENV NS2B-NS3\textsubscript{pro}, binding energies for nimbin, desacetylnimbin and desacetylsalannin were –5.56, –5.24 and –3.43 kcal/mol, respectively. This provides the evidence that the ligand molecules have more affinity to the active site of protein and can be used as efficient and potential inhibitor or drug molecule.

\textit{Interaction analysis of protein-ligand complexes}

The characterization of interactions in protein-ligand complexes is essential for research in the area of structural bioinformatics and drug discovery\textsuperscript{40}. It is crucial for complete understanding of the molecular mechanisms of biological systems\textsuperscript{41}. The interaction analysis of NS2B-NS3\textsubscript{pro} and nimbin complex (Fig. 2) revealed that four residues of DENV NS2B-NS3\textsubscript{pro}, viz. His51, Asp75, Ser135 and Asn152 are involved in the formation of hydrogen bonds between nimbin and NS2B-NS3\textsubscript{pro}, while six residues, viz. Val36, Arg73, Pro132, Gly133, Gly153 and Val154 residues are involved in the hydrophobic interactions. Hydrogen bonding and hydrophobic interactions are key players in stabilizing energetically-favoured ligands, in an open conformational environment of protein structures\textsuperscript{42}. However, it is still poorly understood how the binding parameters associated with these interactions facilitate a drug-lead to recognize a specific target and improve drug’s efficacy\textsuperscript{43–44}. The interaction plot of NS2B-NS3\textsubscript{pro} and desacetylnimbin complex as shown in Fig. 3, illustrated that in total four hydrogen bonds are involved between desacetylnimbin and NS2B-NS3\textsubscript{pro}.

\textbf{Figs. 1 (a–e):} 2D structures of neem triterpenoids from PubChem database: (a) azadirachtin; (b) desacetylnimbin; (c) desacetylsalannin; (d) nimbin; and (e) salannin.

\textbf{Fig. 2:} 2D interaction plot of DENV NS2B-NS3\textsubscript{pro} and nimbin complex generated through Ligplot programme.

\textbf{Fig. 3:} 2D interaction plot of desacetylnimbin and DENV NS2B-NS3\textsubscript{pro} complex.
pocket residues, Arg54, Gly133 and Asn152 respectively, in which all residues formed only one hydrogen bond except Arg54 which formed two hydrogen bonds. In total 8 amino acid residues; Val36, Trp50, His51, Val72, Arg73, Asp75, Pro132, and Ser135 of NS2B-NS3 pro pocket were found to be involved in hydrophobic interaction with desacetylnimbin. From Fig. 4 of DENV NS2B-NS3 pro and desacetylsalannin complex, it can be deduced that only hydrophobic interactions were found between NS2B-NS3 pro and desacetylsalannin. Seven residues (Trp50, His51, His54, Val72, Arg73, Asp75 and Asn152) of the NS2B-NS3 pro pocket were found to be involved in the formation of these weak interactions.

DISCUSSION

Dengue is a complicated arthropod-borne viral infection with wide spectrum of clinical signs and symptoms in humans. Each year, there are ~50 million dengue infections and ~500,000 individuals are hospitalized with dengue haemorrhagic fever, mainly in Southeast Asia, the Pacific and the Americas. Increase in DENV cases is an important medical issue for which neither a specialized vaccine nor an efficient drug available in the market. NS2B-NS3 protease of DENV plays a vital role in genome replication and viral RNA synthesis, through the cleavage of the viral polyprotein precursor to discharge individual non-structural macromolecules and a C-termini- nal NTPase-dependent RNA helicase. Hence, NS2B-NS3 protease is considered as a therapeutic target for the discovery and designing of potential lead compounds to combat the DENV infection. The hydrogen bonding between the ligand and one of the catalytic triad (His-51, Asp-75, and Ser-135) residues of NS3 protease could interrupt electron transfer between the carboxyl group of Asp-75 and nitrogen atom on imidazole group of His-51, possibly disrupting the ability of His-51 to activate nucleophilic attack of hydroxyl group (β-OH) of Ser-135, which is essential for the initiation of proteolysis. Five putative substrate binding residues (Asp129, Phe-130, Tyr-150, Asn-152 and Gly-153), conserved among the Flavivirus, form the small part in the β-sheet which is common in all serine proteases.

In this study, triterpenoids nimbin and desacetyl-nimbin showed hydrogen bond interactions with the catalytic triad and some of the substrate binding residues while the desacetylsalannin showed only hydrophobic interactions with the catalytic triad and some of the substrate binding residues. In an earlier study, it has been reported that the triterpenoids and other active compounds present in the neem plant have potential antiviral activity against coxsackie B group of viruses. In an in silico study, nimbin has been discovered as effective against the envelope protein of four serotypes of DENV (dengue1–4). In another in vitro study, the antiviral activity of triterpenoid saponin, isolated from a Brazilian plant (s21) has been described against herpes simplex virus type I. Findings of the present study suggest that nimbin, desacetyl-nimbin and desacetylsalannin triterpenoids commonly found in neem plant, possess very good binding affinity with DENV NS2B-NS3 pro and can be considered as potential inhibitors of DENV NS2B-NS3 pro for the development of highly effective and potential drugs against dengue virus infection.

CONCLUSION

The present study was undertaken to find out the in silico inhibitory potential of five triterpenoids (nimbin, desacetyl-nimbin, desacetylsalannin, salannin, and azadirachtin) found in neem plant against DENV NS2B-NS3 pro. This study revealed nimbin, desacetyl-nimbin and desacetylsalannin as three potential broad-spectrum inhibitors against DENV NS2B-NS3 pro. The findings attained through this study on the molecular interaction mode of three neem triterpenoids and DENV NS2B-NS3 pro can be considered for further in vitro and in vivo validation for designing new potential drugs against DENV infection. The above study is highly significant,
as till now there is no efficient medicine available to combat dengue infection.

Conflict of interest
All the authors declare that they have no conflict of interest.

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