VirusDis. (October–December 2015) 26(4):243–254
DOI 10.1007/s13337-015-0280-x

ORIGINAL ARTICLE

Computational analysis reveal inhibitory action of nimbin against dengue viral envelope protein

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Received: 3 May 2014 / Accepted: 16 September 2015 / Published online: 20 November 2015

Abstract Dengue has emerged to be global health problem worldwide. Hence there is an immediate need to adopt new strategies in the development of effective anti-dengue drugs. Extracts from the leaves of *Azadirachta indica* has been traditionally used in folk medicine for viral infections. In the present study we report the anti-viral potency of nimbin, the active compound from the neem leaf extract against the envelope protein of dengue virus. Progression of viral entry into the host cell is facilitated by the envelope protein of dengue virus, suggesting; it as an effective anti-viral target. Nimbin is found to be effective against the envelope protein of all four types of dengue virus (dengue 1–4), which is evident from our in silico analysis. Our findings suggest the clinical importance of nimbin, which can serve as effective lead compound for further analysis.

Keywords Dengue · *Azadirachta indica* · Nimbin · Envelope · ADMET

Introduction

In accordance with World Health Organization (WHO) report, more than 50 million people are falling prey to dengue virus every year [18]. Dengue virus belongs to the family; where the mode of transmission is mainly via arthropod vectors [17]. The virus responsible for classical dengue fever, dengue shock syndrome (DSS) and dengue hemorrhagic fever (DHF) is divided into four classes based on the degree of sequence identity [20]. Classical dengue can leads to fatal conditions such as DSS and DHF when subjected to secondary infection by other serotypes. Primary infection is characterized by modest indications like fever associated with joint and muscle pain, whereas secondary infection is characterized by skin hemorrhages, damage to blood and lymph vessels leads to increase in fatal outcome rate. Dengue virus infection is considered to be fatal to the individuals with respiratory problems and other chronic diseases [13]. Envelope of dengue virus facilitates the adherence into host tissues and also responsible for the antigenic property of virus, hence considered as effective target for the development of anti-viral drugs [56]. Previous studies revealing the anti-viral properties of natural products disclose the importance of plants extracts against deadly viruses [7, 27, 52]. *Azadirachta indica,* a medicinal plant found to posses anti-viral property against dengue virus, small pox virus and polio virus as reported by several investigators [39, 44, 51]. At present plants are the indirect or direct sources of approximately 50 % of approved drugs [26]. Since plant derived compounds are of considerably smaller molecules and contain thousands of naturally occurring components, human body can quickly absorb and utilize these compounds. Plants have been traditional source of active substances for most therapies [3]. As per the report published by annual reports of medicinal chemistry, seven out of ten synthetic agents approved by FDA are modeled on a natural product [48]. Neem plant has been widely used as a traditional medicine for many centuries in tropical countries. Several researchers have stressed the

Electronic supplementary material The online version of this article (doi:10.1007/s13337-015-0280-x) contains supplementary material, which is available to authorized users.

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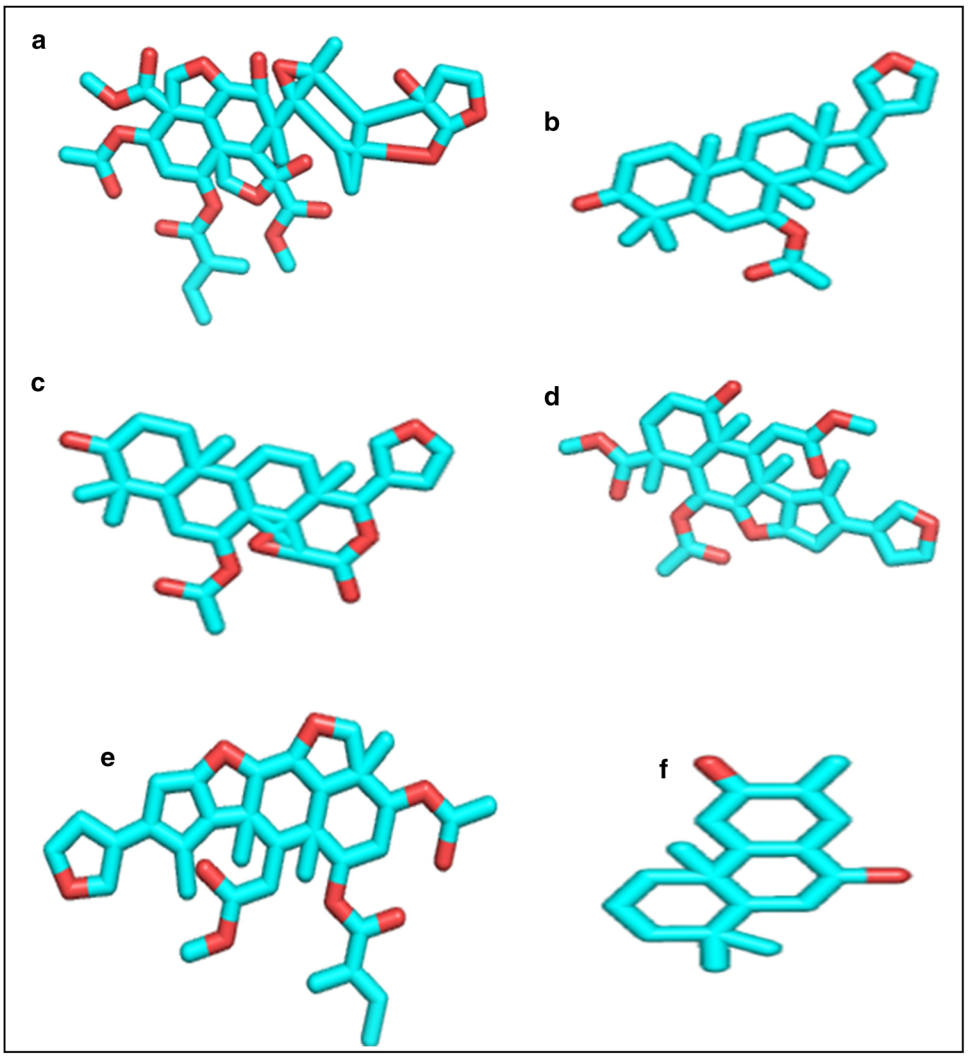

Fig. 1 3D structure of active compounds from the leaves of *Azadirachta indica.* **a** Azadirachtin. **b** Azadirone. **c** Gedunin. **d** Nimbin. **e** Salannin and **f** Ninbidol

anti-viral properties of neem against dengue virus, polio virus, HIV, coxsackie B group virus [39, 43, 44, 45, 47, 54]. In addition the virucidal activity of neem extract is proved against coxsackie B group virus in the early event of its replication cycle. Similarly experimental evidences by Parida et al. [39] proves the inhibitory potential of neem extract at the replication step of dengue virus. Recently, in vitro anti-viral activity of neem bark extract is proved against herpes simplex virus type-1 infection [53]. These evidences strongly support the significance of natural products in the development of anti-viral agents. In the present study, we report the anti-viral potential of neem leaf extracts against dengue virus envelope protein as confirmed by docking analysis and absorption, distribution, metabolism, excretion and toxicity (ADMET) studies.

Materials and methods

Active compounds

Based on literature reports [34, 42] the 3D structures of Nimbin, Ninbidol, Gedunin, Salannin, Azadirachtin and Azadirone from the leaves of *Azadirachta indica,* were retrieved from PubChem database and have been shown in Fig. 1.

Receptor

Dengue virus envelope plays a critical role in the dengue virus fusion with host cell membrane and thus can be considered as potential anti-dengue target. Structure of envelope protein for all the four serotypes were retrieved

Table 1 Binding affinity of active compounds towards the envelope of DENV-1 virus

S. No	Compounds	CScore[a]	Crash score[b]	Polar score[c]	G score[d]	PMF score[e]	D score[f]	Chem score[g]	No. of hydrogen bonds
1.	Nimbin	3.68	−1.14	2.33	−100.07	−72.670	−149.12	−17.988	3
2.	Gedunin	3.46	−1.63	1.19	−110.53	−41.870	−195.83	−13.662	2
3.	Azadirone	2.90	−0.95	0.21	−78.466	−38.585	−163.55	−12.366	3
4.	Salannin	1.95	−1.11	1.22	−133.36	−69.767	−177.73	−21.510	2
5.	Ninbidol	1.72	−1.09	1.78	−344.08	−97.851	−150.06	−22.381	1
6.	Azadirachtin	3.33	−1.75	2.19	−115.23	−40.890	−156.32	−14.335	3
7.	Panduratin*	2.33	−1.15	1.35	−65.92	−27.512	−158.31	−11.266	1

* Reference ligand

[a] CScore is a consensus scoring which uses multiple types of scoring functions to rank the affinity of ligands

[b] Crash-score revealing the inappropriate penetration into the binding site

[C] Polar region of the ligand

[d] G-score showing hydrogen bonding, complex (ligand–protein), and internal (ligand–ligand) energies

[e] PMF-score indicating the Helmholtz free energies of interactions for protein–ligand atom pairs (Potential of Mean Force, PMF)

[f] D-score for charge and van der Waals interactions between the protein and the ligand

[g] Chem-score points for hydrogen bonding, lipophilic contact, and rotational entropy, along with an intercept term

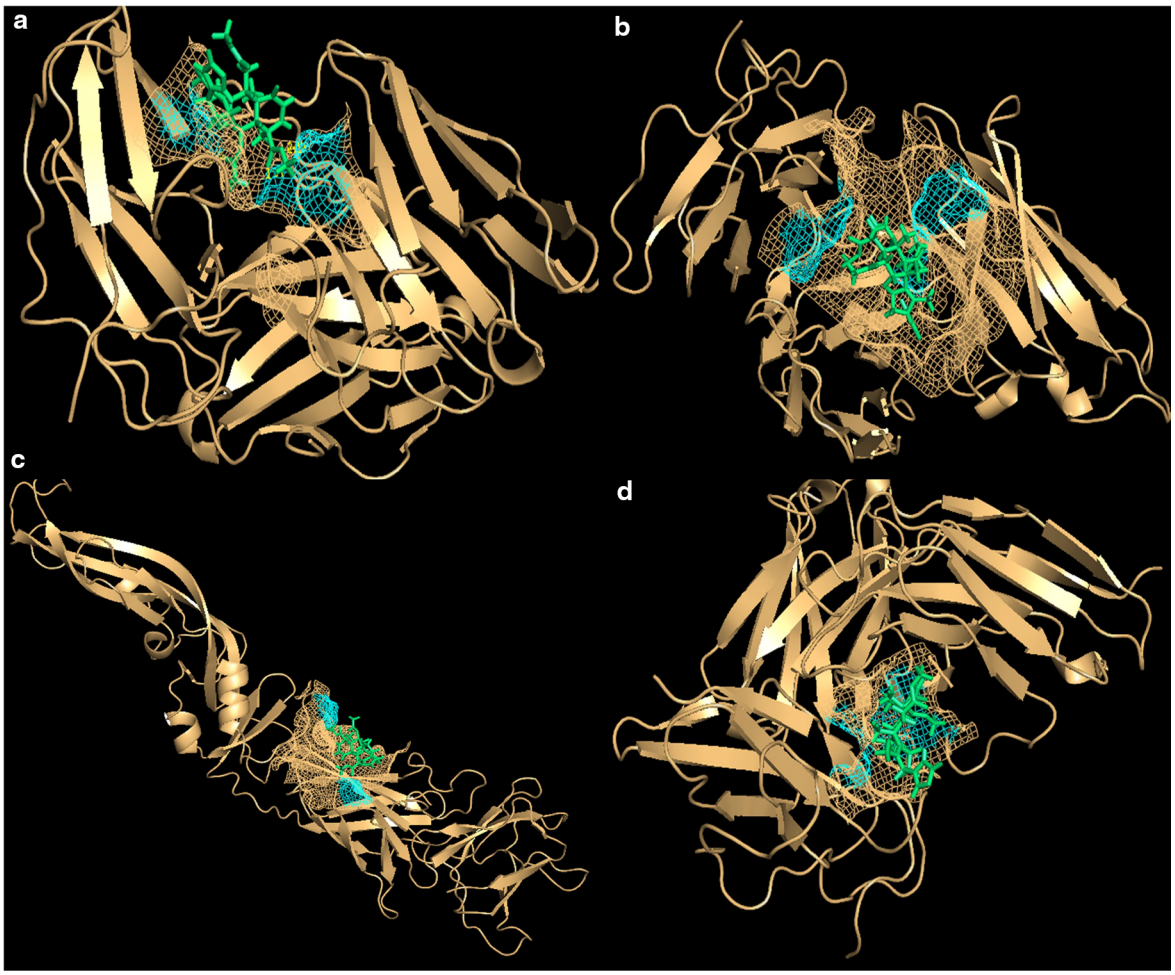

Fig. 2 Docked structure of the compound Nimbin in the active site of **a** DENV-1. **b** DENV-2. **c** DENV-3 and **d** DENV-4. Active site of the receptor is shown in mesh representations

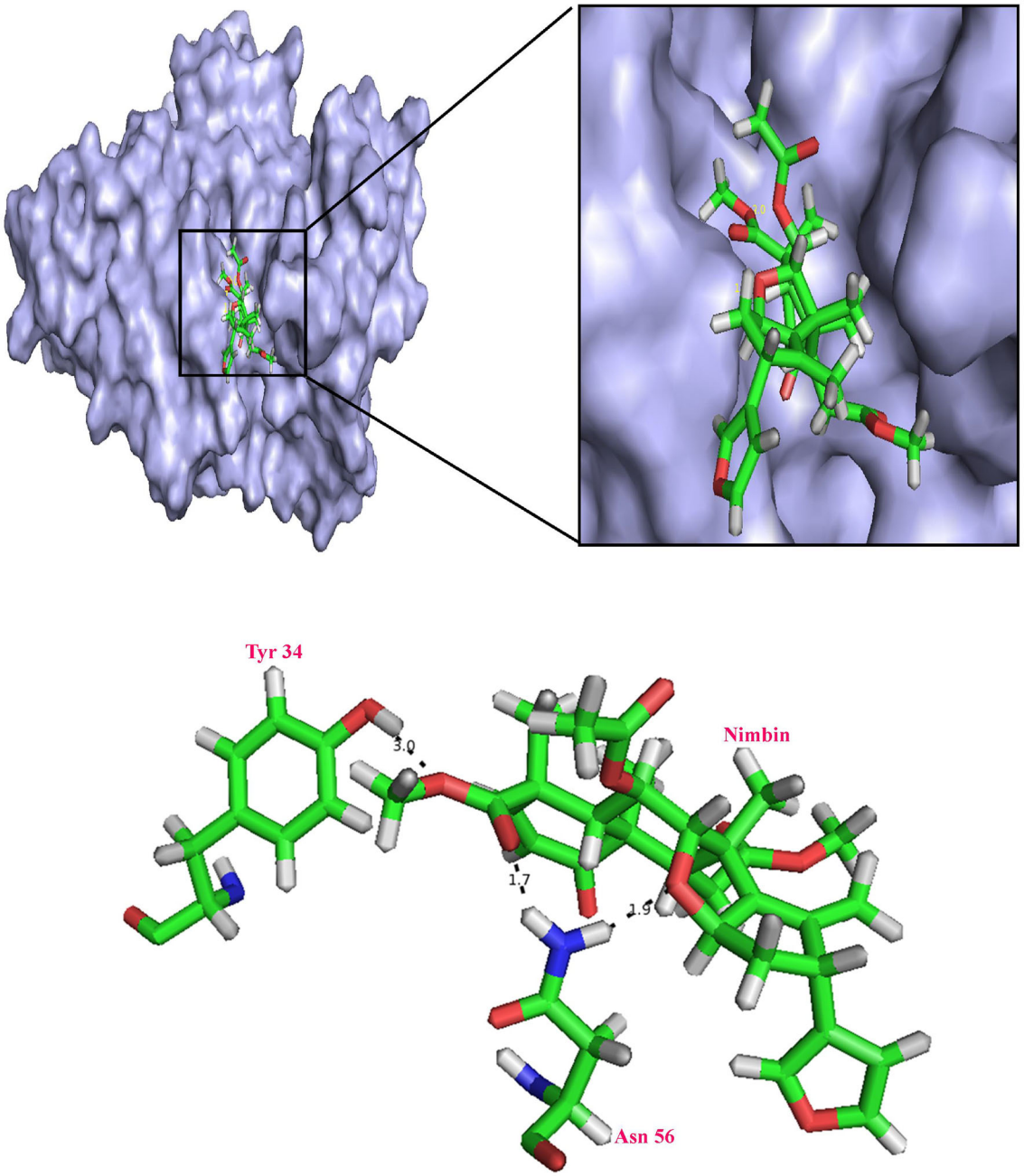

Fig. 3 Pictorial representation hydrogen bond interactions between nimbin and the active site residues of DENV-1. *Dotted lines* represents the hydrogen bonds interactions between H atom of Tyr 34 with O atom of nimbin, HD2 atom of Asn 56 with O atom of nimbin and H atom of Asn 56 with O atom of nimbin

by Protein Data Bank (PDB) [4]. The PDB ID's of chosen structures are shown below.

I. 3UZQ—DENV1envelope [11]
II. 1TG8—DENV2 envelope [58]
III. IUZG—DENV 3 envelope [35]
IV. 3UYP—DENV 4 envelope [24]

Molecular docking studies

Docking analysis was performed with Surflex-dock program incorporated in SYBYL 2.0 [23]. The surflex dock scoring function is based on Hammerhead docking system [28] and it effectively neglects the false positive results,

Table 2 Binding affinity of active compounds towards the envelope of DENV-2 virus

S. No	Compounds	CScore[a]	Crash score[b]	Polar score[c]	G score[d]	PMF score[e]	D score[f]	Chem score[g]	No. of hydrogen bonds
1.	Nimbin	3.26	−1.11	0.01	−90.30	−10.14	−191.47	−15.507	4
2.	Azadirone	3.15	−1.11	0.01	−93.30	−15.143	175.23	−21.252	2
3.	Gedunin	3.11	−2.52	2.03	−104.87	−19.18	−206.00	−20.252	2
4.	Salannin	1.49	−1.34	1.47	−272.24	−28.690	−155.09	−22.227	1
5.	Ninbidol	1.44	−0.86	1.85	−206.94	8.424	−149.46	−20.544	1
6.	Azadirachtin	3.23	−1.10	0.01	−95.8	−13.52	−190.47	−16.206	4
7.	Panduratin*	1.25	−0.95	1.15	−205.91	−17.512	−138.32	−20.266	1

* Reference ligand

[a] CScore is a consensus scoring which uses multiple types of scoring functions to rank the affinity of ligands

[b] Crash-score revealing the inappropriate penetration into the binding site

[C] Polar region of the ligand

[d] G-score showing hydrogen bonding, complex (ligand–protein), and internal (ligand–ligand) energies

[e] PMF-score indicating the Helmholtz free energies of interactions for protein–ligand atom pairs (Potential of Mean Force, PMF)

[f] D-score for charge and van der Waals interactions between the protein and the ligand

[g] Chem-score points for hydrogen bonding, lipophilic contact, and rotational entropy, along with an intercept term

thus helps in screening of the active compounds effectively. The Structure obtained from PDB is optimized by removing the co crystallized structures, crystallographic water molecules and other unrelated structures. Using the force field AMBER 7 FF99, the structure is subjected to brief minimization after the addition of hydrogen atoms. Surflex-Dock protomol is referred to as 'binding pocket', in which the active compounds are aligned. Using empirically derived scoring functions, the binding affinity of ligand and receptor is calculated. To evaluate the binding affinity of ligand-receptor interactions Consensus scoring (CScore) function were used. CScore combine the use of several scoring functions to obtain the efficient results and to avoid the disadvantages of other scoring functions. CScore is in the combination of Gold score (GScore), Dock score (DScore), Potential Mean force score (PMF score), polar score and crash score. GScore [33] and DScore [12] were used to calculate energy of hydrogen bond interaction and hydrophobic and electrostatic interactions respectively. Also the energy from lipophilic interactions, hydrogen bonding interactions was calculated using Chemscore [36]. To observe the interaction between ligand and receptor, Molecular Computer Aided Design (MOLCAD), a visualization tool is used.

Calculation of molecular properties

The tools Molinspiration and admetSAR were used to calculate the molecular properties of the active compound [9, 50]. ADMET properties include blood–brain barrier (BBB), Human intestinal absorption (HIA), Caco-2 Permeability, p-glycoprotein inhibitor, Renal organic cation transporter, CYP450 (Cytochrome P450) 2C9 Substrate, CYP450 2D6 Substrate, CYP450 3A4 Substrate, CYP450 1A2 Inhibitor, CYP450 2C9 Inhibitor, CYP450 2D6 Inhibitor, CYP450 2C19 Inhibitor, CYP450 3A4 Inhibitor and AMES Toxicity. Further, the compound nimbin is tested for their cardiotoxicity risks using the tool LabMol Pred-hERG 2.0 [5, 6].

Results

Molecular docking studies

The envelope of DENV serve as an important anti-viral target, hence docking analysis is performed on a series of active compounds against the envelope of all four DENV. In order to identify the efficiency of active compounds, our results were also compared with the reference ligand Panduratin [14]. Table 1 shows the energy of binding affinity of all the compounds against DENV-1 virus. From the results we observe that compound Nimbin shows maximum binding affinity than other compounds. Even though Azadirone and Azadirachtin form three hydrogen bonds with the active site residues, they show poor results in protein-ligand atom pair interactions. CScore of Gedunin is in the same range of Nimbin but the number of hydrogen bond interaction formed is comparatively less. Since the hydrogen bond formation makes an important contribution

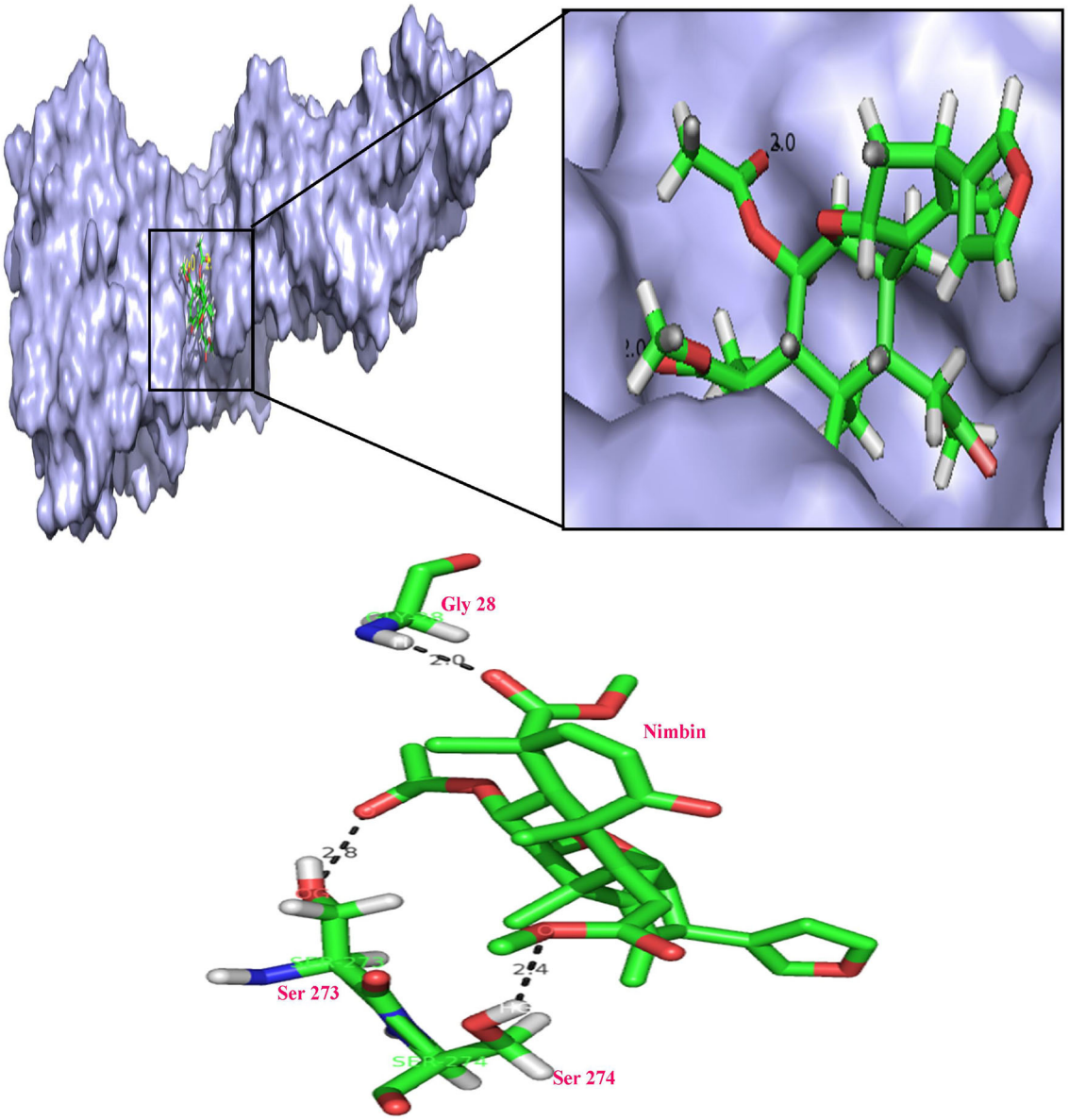

Fig. 4 Pictorial representation hydrogen bond interactions between nimbin and the active site residues of DENV-2. *Dotted lines* represents the hydrogen bonds interactions between H atom of Gly 28 with O atom of nimbin, OG atom of Ser 273 with O atom of nimbin and HG atom of Ser 274 with O atom of nimbin

in protein-ligand interactions, the number of hydrogen bonds formed between the active compounds and the receptors are also listed in Table 1. Docked structure of Nimbin in the active site of all four serotypes is shown in Fig. 2. Further the hydrogen bonding interaction between the compound Nimbin and the active site residues of DENV-1 are depicted in Fig. 3. On analyzing the results, we observe that the compound Nimbin and Azadirachtin shows highest binding affinity with a maximum of four hydrogen bonds. Table 2 shows the energy of binding affinity of active compounds towards the envelope of DENV-2 virus. Pictorial representation of hydrogen bonding interaction between Nimbin and the active site residues of DENV-2 are shown in Fig. 4. Even though azadirone and azadirachtin shows equal polar interaction, the number of hydrogen bonds and CScore is compara-

Table 3 Binding affinity of active compounds towards the envelope of DENV-3 virus

S. No	Compounds	CScore[a]	Crash score[b]	Polar score[c]	G score[d]	PMF score[e]	D score[f]	Chem score[g]	No. of hydrogen bonds
1.	Nimbin	3.79	−0.76	2.17	−76.666	−15.983	−165.24	−16.325	3
2.	Gedunin	2.69	−1.00	0.99	−70.787	−30.324	−173.05	−12.388	3
3.	Azadirone	2.50	−1.32	0.96	−94.813	−25.101	−178.37	−17.401	3
4.	Ninbidol	1.20	−0.92	1.11	−210.08	−7.003	−155.32	−22.227	2
5.	Salannin	1.07	−1.21	0.44	−235.45	−34.09	−176.33	−19.688	1
6.	Azadirachtin	2.65	−1.05	0.95	−80.236	−28.411	−160.58	−15.603	2
7.	Panduratin*	2.15	−1.05	1.15	−85.91	−27.422	−168.51	−12.254	2

* Reference ligand

[a] CScore is a consensus scoring which uses multiple types of scoring functions to rank the affinity of ligands

[b] Crash-score revealing the inappropriate penetration into the binding site

[C] Polar region of the ligand

[d] G-score showing hydrogen bonding, complex (ligand–protein), and internal (ligand–ligand) energies

[e] PMF-score indicating the Helmholtz free energies of interactions for protein–ligand atom pairs (Potential of Mean Force, PMF)

[f] D-score for charge and van der Waals interactions between the protein and the ligand

[g] Chem-score points for hydrogen bonding, lipophilic contact, and rotational entropy, along with an intercept term

tively low than nimbin. Since hydrogen bond act as an important contributor to free energies of biological macromolecules and macromolecular complexes, it plays an important role in determining the binding affinity of ligands. Considering the energetic contribution through van der Waals interactions the compound Gedunin shows the maximum score, but the number of hydrogen bonds formed between Gedunin and the active site residues are less comparing to Nimbin and Azadirone. Finally Salannin and Ninbidol shows the least score among the six compounds analysed indicating the poor binding affinity between the ligand and the DENV-2 virus envelope. Table 3 shows the binding affinity of active compounds and the DENV-3 virus envelope. Figure 5 represents hydrogen bonding interaction between the compound Nimbin and the active site residues of DENV-3. From the results we find that the compound Nimbin and Gedunin dominates with a maximum of three hydrogen bonds. Even though Azadirone shows maximum hydrogen bond interaction, the total score is comparatively less than Nimbin and Gedunin. Salannin and Ninbidol shows poor values in all seven parameters analysed. Results of binding pattern of DENV-4 virus are depicted in Table 4 and the hydrogen bond interactions are shown in Fig. 6. It is noteworthy to mention that Nimbin shows maximum binding affinity with DENV-4, similar to DENV-1 DENV-2 and DENV-3, whereas Salannin shows poor energy values in all the parameter analyzed. It is also worth mentioning that nimbin shows high binding affinity comparing to panduratin in all the four complexes analyzed. These results reveal the significance of nimbin as a potent anti-viral compound.

ADMET profiling

The physiochemical property and biological activity of the active compound Nimbin is analyzed based on Lipinski's rule of 5. Our analysis shows that the compound Nimbin successfully passed through the Lipinski's filter with minor violation. The results of the molecular properties and drug likeness of Nimbin is presented in Supplementary Table 5. Further ADMET profiling of Nimbin is performed and the results are tabulated in Supplementary Table 6. ADMET profiling is carried out for the parameters including blood–brain barrier penetration, Caco2 permeability, p-glycoprotein inhibitor, renal organic cation transporters and toxicity studies. Further predicted hERG risk for active compound shows favorable results which reveal the efficiency of nimbin to act as drug candidate.

Discussion

Currently no effectual treatment is available to conflict the dengue viral infection therefore development of effective medicine without any adverse side effects is necessary [19]. Hence in the present study we have explored anti-viral property of active compounds from *Azadirachta indica* through docking studies. Studies on natural compounds

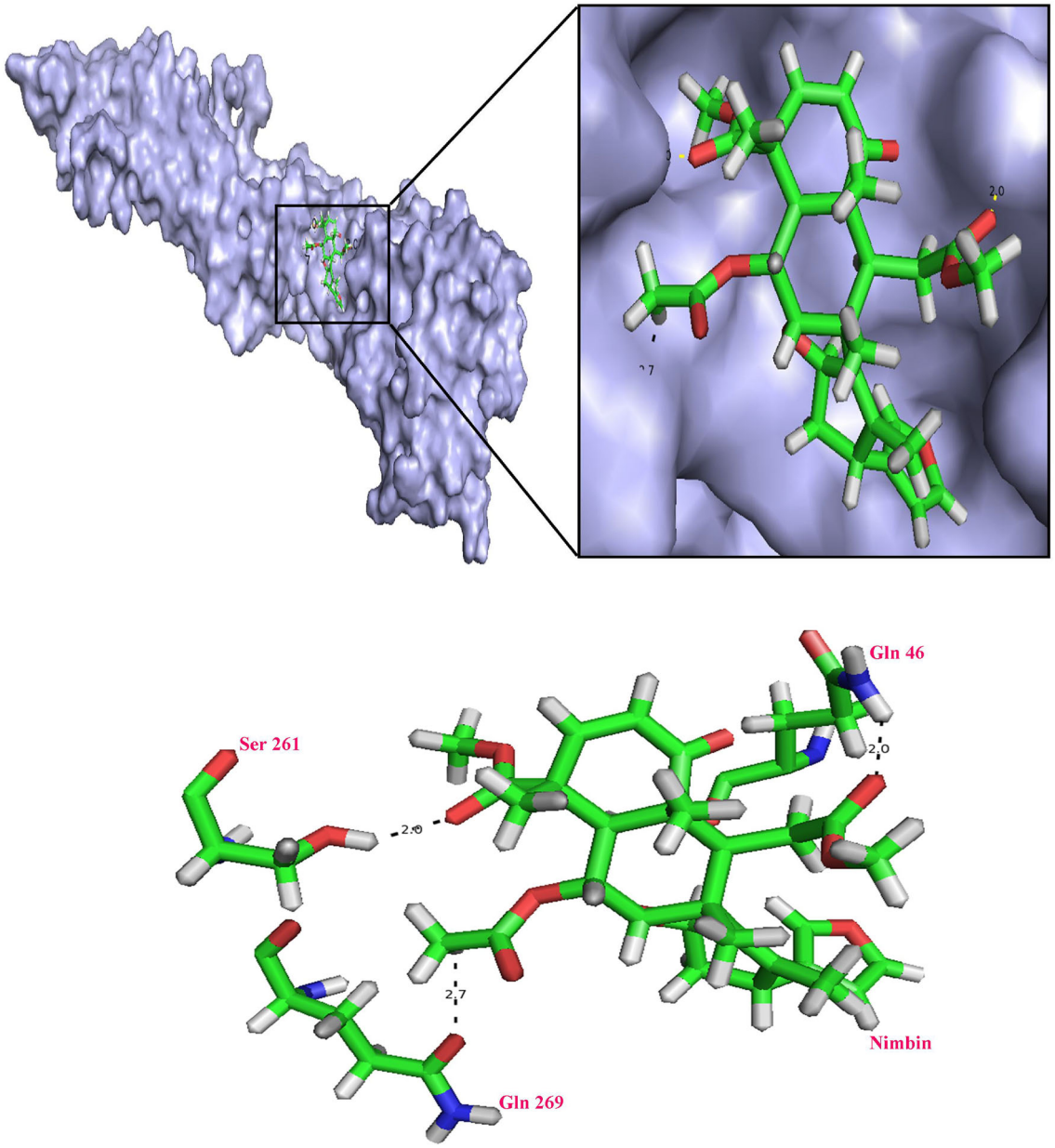

Fig. 5 Pictorial representation hydrogen bond interactions between nimbin and the active site residues of DENV-3. *Dotted lines* represents the hydrogen bonds interactions between HG atom of Ser 261 with O atom of nimbin, OE1 atom of Gln 269 with H atom of nimbin and HE1 atom of Gln 46 with O atom of nimbin

reveal the anti-viral property of plant derived products [29]. It is also interestingly reported that higher plants exhibits greater inhibitory property against viruses [2]. Anti-viral properties of *Azadirachta indica* is reported by several investigators [15, 39]. Presence of polyphenolic substances in the leaves of *Azadirachta indica* is responsible for the pharmacological properties of neem trees [49]. Our analysis reveals that active compound Nimbin shows high binding affinity against the DENV envelope. Nimbi is the major compound found in *Azadirachta indica* is reported to elucidate the pesticida properties of neem leaf extracts [55].

As proved in earlier studies "Rule of Three" could be useful when constructing fragment libraries for efficient lead discovery [46]. Our study involves the evaluation of the conformations of the whole ligand without ligand fragmentation. Docking of the ligands to the target was

Table 4 Binding affinity of active compounds towards the envelope of DENV-4 virus

S. No	Compounds	CScore[a]	Crash score[b]	Polar score[c]	G score[d]	PMF score[e]	D score[f]	Chem score[g]	No. of hydrogen bonds
1.	Nimbin	3.37	−0.77	1.10	−85.944	13.383	−174.23	−18.83	4
2.	Azadirone	3.11	−0.99	1.28	−92.730	1.031	−162.25	−11.90	2
3.	Gedunin	3.06	−0.76	2.72	−83.563	0.991	−144.51	−18.825	3
4.	Ninbidol	1.04	−1.20	2.62	−347.54	8.945	−128.58	−21.90	2
5.	Salannin	0.71	−1.62	1.25	−172.65	10.358	−163.34	−23.687	1
6.	Azadirachtin	3.05	−0.82	1.35	−87.378	2.274	−158.75	−16.423	4
7.	Panduratin*	1.75	−1.27	2.17	−285.31	7.425	−159.27	−22.584	1

* Reference ligand

[a] CScore is a consensus scoring which uses multiple types of scoring functions to rank the affinity of ligands

[b] Crash-score revealing the inappropriate penetration into the binding site

[C] Polar region of the ligand

[d] G-score showing hydrogen bonding, complex (ligand–protein), and internal (ligand–ligand) energies

[e] PMF-score indicating the Helmholtz free energies of interactions for protein–ligand atom pairs (Potential of Mean Force, PMF)

[f] D-score for charge and van der Waals interactions between the protein and the ligand

[g] Chem-score points for hydrogen bonding, lipophilic contact, and rotational entropy, along with an intercept term

performed using a whole molecule approach, as described previously [10, 25]. Hence physiochemical property and biological activity of the active compound Nimbin is analyzed based on Lipinski's rule of 5. As a general rule, molecular weight of the compound should be not more than 500 daltons. Even though molecular weight of the compound Nimbin isgreater than 500 daltons it can exempted from violation as per the rules stated by GlaxoSmithKline (GSK) groups [30]. This rule states that the compound with molecular weight >500 but with constrained polar surface (PSA) may also show good oral bioavailability [41]. According to the earlier reports [8] compounds with polar surface area of >140 Å leads to poor oral bioavailability, since the PSA of Nimbin is below the cut off value, the compound should be considered to have good oral bioavailability. Pharmacokinetic analysis on drug discovery states that molecular weight of the drug within the range of 150 and 550 Da exhibits good bioavailability [1, 8]. Physiochemical properties of compound determine the capability of drug to pass the blood brain barrier [16]. Since dengue infection is also associated with central nervous system [38], the drug should possess the capability to cross blood brain barrier and exhibit CNS activity. Compounds within the molecular range of 400–600 Da are efficiently transported through the blood–brain barrier [37]. The BBB efficiency of the compound Nimbin is analysed and our reports shows the positive results. Polar surface area of the compound efficiently determines the intestinal absorption of drugs [21]. As stated earlier, PSA value of Nimbin is within the threshold indicates the greater absorption of the compound. In earlier studies it has been proved that esters or amides are introduced to the drug molecule and hence enhance the aqueous solubility [31, 32]. An ester functional group has the potential to interact with binding site as a hydrogen bond acceptor. Esters are susceptible to hydrolysis in vivo by metabolic enzymes called esterases, which may decrease the life span of the drug in vivo. Even though esterase plays an important role in drug metabolism, there is a strong support from experimental studies which proves that those ester groups in drugs are stabilized by electrostatic and steric stabilization [40]. Intestinal esterase acts on ester type drugs administered orally at the beginning of ester type drug metabolism. It is also proved that pharmacological effects were influenced by the hydrolyzing activity of intestinal esterase [22, 57]. It is interestingly observed that the active compound Nimbin consists of three ester moieties, which can leads to increased absorption and oral bioavailability of compound. As proved in earlier studies presence of ester groups in drugs increases the absorption and oral bioavailability of compound [31, 32]. Human intestinal absorption of drugs can be effectively predicted using Caco2 cells. In silico prediction of Caco2 permeability of Nimbin shows good correlation with the human intestinal absorption. Metabolism of drugs and other foreign particles is carried out by the enzyme cytochrome P450. It is also reported that majority of the drugs are metabolized by the following isoforms, CYP1A2, CYP2C9, CYP2C18, CYP2C19,

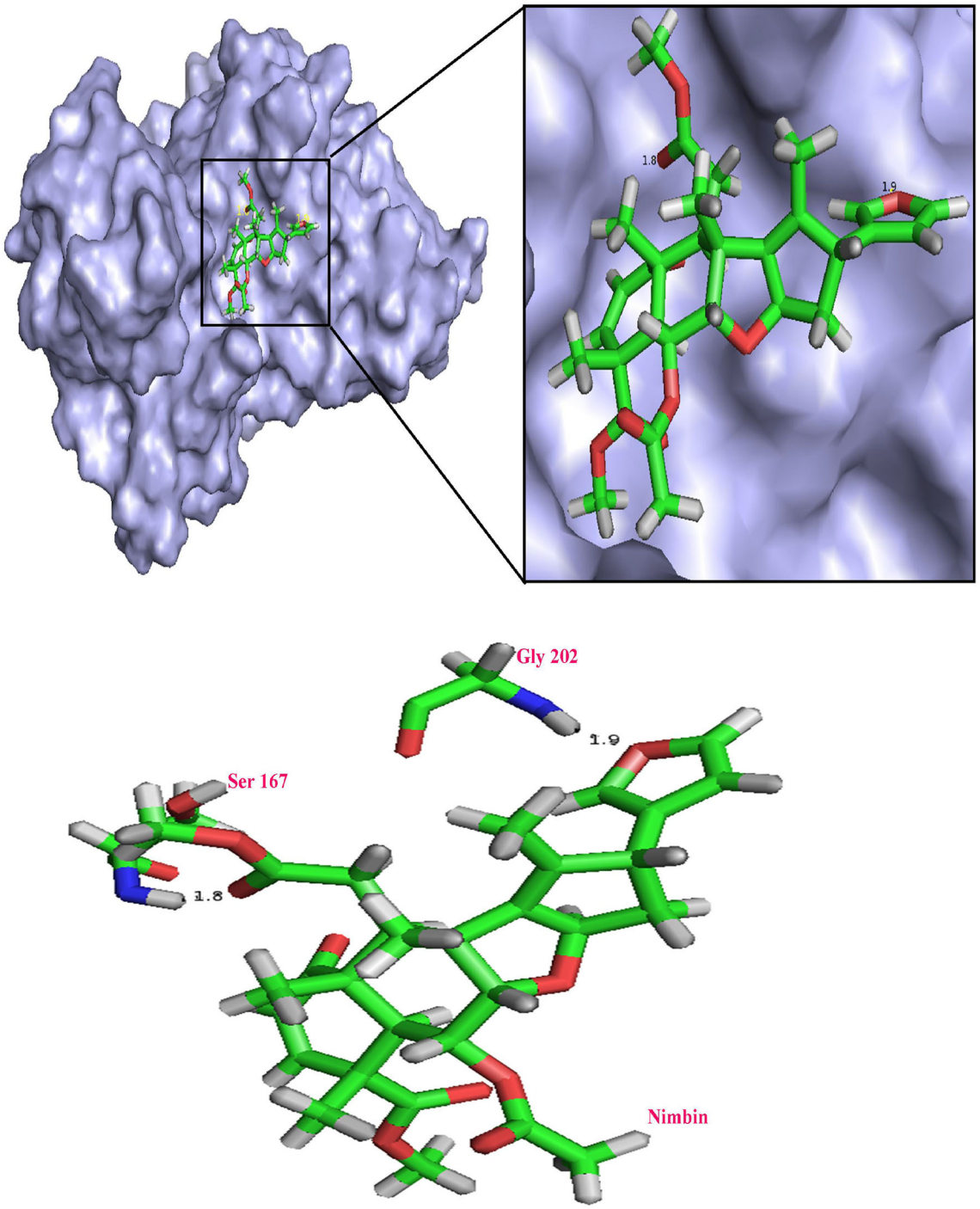

Fig. 6 Pictorial representation hydrogen bond interactions between nimbin and the active site residues of DENV-4. *Dotted lines* represents the hydrogen bonds interactions between H atom of Ser 167 with O atom of nimbin and H atom of Gly 202 with O atom of nimbin

CYP2D6, CYP2E1 and CYP3A4. ADMET properties of Nimbin on different models such as BBB penetration, P-glycoprotein substrate, renal organic cation transporter, human intestinal absorption and Caco2 permeability shows positive results suggesting Nimbin as an effective anti-dengue compound. On the whole we conclude that further optimization of Nimbin can contribute significantly in reducing the morbidity and mortality of dengue infection.

Acknowledgments S.R and A.A gratefully acknowledges the Indian Council of Medical Research (ICMR), Government of India Agency for the research Grant [IRIS ID: 2014-0099] to carry out this research. P. Lavanya thanks ICMR for the research fellowship. We

would like to thank the management of VIT University for providing us the necessary facilities to carry out this research project.

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