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Towards the Identification of Novel Phytochemical Leads as Macrodomain Inhibitors of Chikungunya Virus Using Molecular Docking Approach

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ABSTRACT

The outbreak of Chikungunya fever worldwide has raised a threat to human health and an economic burden on the affected countries. Currently, there is no cure for Chikungunya. The computational methods assist in identifying the drug leads for the target. This work reports the potential phytochemical inhibitors against a unique macro domain present in the conserved N-terminal region of Non- structural protein nsP3 of Chikungunya virus. Macro domain contributes a lot to viral fusion and replication. The shape based docking protocol was applied to predict the ligand conformations and their relative orientations with 2-ADP Ribose binding site of the target. The phytochemicals showed favorable bonded and non-bonded interactions with crucial amino acids. The binding affinity of the ligands to the target structure was estimated with six well-known scoring functions. An all- atom molecular dynamics simulation was performed for the macro domain in apo form and the complex with the best scoring ligand to study the stability and the conformational flexibility of the complexes. Based on the multiple scoring functions, drug- like properties, amino acid interactions and molecular dynamics studies, it is believed that the phytochemicals screened may well compete with the substrate. This is turn can inhibit the function of macro domain, eventually the loss of virulence of chikungunya virus. The work also gives a room for designing novel drugs using chemoinformatics, based on the structures of phytochemical leads reported in this study.

INTRODUCTION

The emergence of viral diseases has always been a burden to a Country's economy. Viruses co-evolve with Human. The evasion mechanisms adopted by the viruses to protect themselves from the host immune system have left the scientific community to find a novel solution to tackle such situation. One of the well- known viral diseases in recent years across the globe is Chikungunya. This is basically a mosquito borne viral disease caused by Chikungunya virus. Though most of the symptoms are not unique to this disease, it leaves the patient with uncontrollable joint pain even after recovery. At present there is no cure available for this disease. The current treatments are based on relieving the symptoms. The plethora of anti- inflammatory drugs is given as the solution for both fever and inflammation. The research is now focused on finding the newer drug targets in Chikungunya virus. The computer aided drug designing serves as a platform for designing new drugs based on the structure of the protein targets. The computational methods have been applied to the protein targets of chikungunya virus to design novel lead molecules (Rashad and Keller, 2013). The success of any drug discovery process relies on the target selection. Especially with viruses, their evasion mechanisms remain challenging. Hence, the best target could be the one which is highly conserved and most important for their survival.

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The structure of this virus was studied to understand the various proteins involved in its virulence. Structurally, the chikungunya viral genome encodes four major non- structural proteins (nsP 1-4) and three major structural proteins (A capsid and two envelope proteins) (Khan *et al*., 2002). Of these, the function of nsP3 is poorly understood (Han *et al.,* 2011). It is made of two domains, a unique macro domain in the conserved N-terminal region and the less conserved C-terminal region. Macro domain is a highly conserved protein domain found in both eukaryotes and prokaryotes (Neuvonen and Ahola, 2009). These are also encoded by a set of positive-strand RNA viruses that replicate in the cytoplasm of animal cells, including corona viruses and alpha viruses. Although the functions of the macro domain are poorly understood, it has been suggested to be an ADP-ribose-binding module.

Though the role of phosphorylation is not well documented, deletion of the phosphorylated residues showed a decrease of RNA synthesis level (Vihinen, 2001). The absence of phosphorylation on nsP3 in an alpha virus, Semliki Forest virus (SFV) showed decreased viral pathogenecity, and the absence of the C terminus of nsP3 alters SFV neuro- virulence (Tuittila *et al.,* 2000). This shows the importance of phosphorylation in the virulence of the virus especially the alpha virus to which the chikungunya virus also belongs to. Macro domains are thought to be active as adenosine di- phosphoribose 1"-phosphate phosphatases. The binding of the macro domain to ADP Ribose may facilitate the Host- viral fusion, resulting in the transfer of genetic material to the host genome thereby favoring the infection. Any disturbance to this binding may weaken the viral fusion, thus resulting in the loss of virulence. Researchers have determined the structures of these domains of chikungunya virus using crystallography.

There are reports that demonstrate the role of macro domain in viral replication (Vasiljeva *et al.,* 2001). Based on the facts and the outcomes from the various literatures, the macro domain was considered as the target of the study. Our work focuses on projecting the macro domain as an anti- viral drug target and identifying phytochemical leads for treating chikungunya disease.

The phytochemicals have shown their potential abilities in curing viral diseases (Jassim and Naji, 2003). Our aim is to identify the phytochemical leads that could bind to the macro domain of chikungunya virus which competitively inhibit the substrate, thereby affecting the viral fusion. This in turn may control the viral population in the host. In recent years, computational methods are considered as a boon in finding the needle from the haystack (Alam and Khan, 2014). They are both cost and time effective.

The potent phytochemical leads having affinity towards this target were identified using structure based drug designing. Docking based approaches have shown positive results in the identification of the ligands that have been proved experimentally for various targets (Nirmal *et al*., 2015). The important physico-chemical properties of the phytochemicals have also been determined to check for their drug- likeliness character. To study the behavior of ligand in complex with the macro domain, an all-atom molecular dynamics was also performed.

MATERIALS AND METHODS

The crystal structure of Chikungunya virus nsP3 macro domain that defines a conserved adenosine binding pocket (3GPO) was considered as the target of the study (Malet *et al.,* 2009). It was retrieved from the Protein Data Bank (Berman *et al.,* 2000). About 150 phytochemicals from various plant sources that may possess anti- viral effect were considered as ligands and this information was retrieved from Dr. Duke's Database (Duke, 2000).

The 3D structures of the ligands in sdf format were downloaded from PubChem (Bolton *et al.,* 2008). The interactions between the target and the viable phytochemicals were studied using LigandFit (Venkatachalam *et al.,* 2003). It is a shape based method for accurately docking ligands into protein active sites. It uses a cavity detection algorithm for detecting cavities in the protein as candidate active site regions. This method has been used to identify the potential leads for the targets (Jasmine and Vanaja, 2013). The docking method involved the generations of sites in the target. The structure was minimized using CHARMm force field and the putative binding sites were detected using the Eraser algorithm.

The location, volume, and shape of the binding sites are all used by LigandFit to filter incompatible ligands, and to create shape-based alignments of candidate poses. Similarly the ligands were also prepared for their perfection in 3D structures. The duplicates if any were also removed. A Monte Carlo method was employed in the conformational search of the ligand. The conformational search procedure allows for multiple torsions to be changed in a single search step. The values of the torsions depend on the number of rotating atoms. The Receptor- ligand interactions were quantified using a set of six scoring functions: LigScore1, LigScore2, PLP1, PLP2, Jain and PMF. The LigScore functions are empirical scoring that attempt to accurately predict the binding affinity between ligand molecules and their protein receptors (Krammer *et al.,* 2005). LigScore1 uses the partial charges on the atoms of both ligand and receptor to determine whether an atom is polar or non- polar based on a cutoff threshold. This influences the computation of C+Pol and TotPol2.LigScore2 type atoms of ligands and proteins as polar or non- polar based on rules that employ only formal charges, ignoring partial charges. The Piece-wise Linear Potential (PLP) is a fast, simple docking function that has been shown to correlate well with the receptor- ligand affinities.

The scores are in arbitrary units of energy. Two versions of PLP function, PLP1 and PLP2 are available. These scores do not use any charge information, so partial charges, formal charges, and force field have no effect on the computed scores (Parrill and Reddy, 1999). The Jain scoring function depends explicitly on the formal charge values in the polar attractive and repulsive

interaction terms. The function is independent of both partial charges and force field (Jain, 1996). The Potential of Mean Force (PMF) scoring function is a statistical based approach using 3D structure databases to provide a fast and accurate prediction of receptor- ligand binding free energies. It is defines as the sum of the free energies over all inter- atomic pairs of the receptor- ligand complex (Muegge and Martin, 1999). The important physico-chemical properties of the screened ligands were studied using QikProp (Schrödinger, 2015). The macro domain is a folded globular protein. Any change in the flexibility of this protein after binding with the phytochemicals signifies the interactions of the ligands with this protein.

The flexibility of the macro domain and its complexes with the ligands were simulated using an efficient method called CABS- flex (Jamroz *et al*., 2013). The molecular dynamics was performed using GROMACS version 4.5.6 (Apol *et al.,* 2010). The topology files for the ligands to use in molecular dynamics studies were generated using PRODRG server (Schüttelkopf and Van Aalten, 2004). The molecular dynamics was carried out for the macro protein in apo form and its docked complex with galangin to study the behavior of the ligand in comparison with the apo protein. The proteins were minimized using GROMOS96 43a1 force field and any duplicate atoms present were removed, followed by the correction in the missing hydrogen atoms and bonds in the protein structures. The proteins were soaked in SPC explicit water solvent resulting in a total of 35629 and 36451 atoms in a cubic box for apo and galangin bound macro protein respectively.

The topology file for galangin was added to the system. Since the charge of the system was not neutral (+1), a cl^- ion was added to neutralize it. This was followed by the removal of any steric clashes introduced during the process. The maximum number of steps for minimization was set to 50000 using a steepest descent algorithm. All the long ranged non- bonded interactions were treated well during the minimization step. After system relaxation, a constant number, volume, and temperature (NVT) simulation was performed for 100 ps at a temperature of 300 K and the coordinates were saved or every 0.2 ps. After temperature stability was attained, a constant number, pressure, and temperature (NPT) simulation was performed for 100 ps. During this phase of the simulation, the temperature was set to 300 K and the pressure to1 bar, with coupling constant of 0.1 and 0.1 ps respectively.

During the simulations, position restraints were applied to the ligand and the macro domain. The long range non- bonded interactions were treated. The molecular dynamics with this set up was run for 6ns each. A leap frog based molecular dynamics was followed for the apo protein and the protein in complex with the best scoring ligand galangin for a period of 6ns. The molecular dynamics simulations for the apo and docked proteins were analyzed by extracting the information from the trajectory files obtained during the simulations. The important parameters like root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), solvent accessible surface area (SASA), and hydrogen bonds (H-bonds) were analyzed and compared.

RESULTS AND DISCUSSION

For the purpose of docking, the original substrate (2-ADP Ribose) in the target was removed. This was followed by energy minimization using CharmM. The Eraser algorithm used by LigandFit detected four binding pockets in total. These pockets were compared with the original crystal structure to assure the possible position of attachment of ADP- Ribose to the receptor. From the crystal data viewed through Accelrys Discovery Studio Visualizer, it was evident that the binding pocket of ADP- Ribose was extended to sites 1 and 2. Hence, the ligands docking to any of these or both sites may affect the binding of ADP- Ribose to this domain resulting in the loss of function of this protein. Of 4 sites detected, the sites 1 and 2 were assumed to be the binding pockets for the substrate based on the comparison with the crystal data (3GPO). The docking was done between all sites and the phytochemicals to check for the preference of ligands to the binding sites. It was noted that many ligands and their poses were failed to dock in sites 3 and 4, which were away from ADP-Ribose moiety. Hence those sites were not considered for further study. The co-crystal ligand, 2-ADP Ribose was used as a control to check the efficacy of the applied docking protocol. The docking protocol followed was able to position the ligand in the pocket that was similar to the information given in the crystal data. Hence it was assured that the given protocol was appropriate for the study. A maximum of 10 poses was retained for every ligand. Out of 150 ligands, 978 poses were docked and 47 poses were failed to dock in site 1. In 2^{nd}site, 771 poses were docked and 30 poses were failed to dock. For some phytochemicals, all ten poses were docked to the sites. The structure based docking showed the preference of ligands to the receptor target. The receptor- ligand interactions were evaluated on the basis of various scoring functions and the amino acid interactions in the site. The ranges of the scores of the ligands bound to site 1 and 2 are tabulated (**Table 1**).

Table 1: The range of scores of ligands docked to two different sites.

Scoring Functions	Site 1	Site 2
LigScore1	-0.52 to 5.94	-0. 25 to 4.9
LigScore2	0.59 to 6.32	1.06 to 5.26
PLP1	-30.63 to -112.58	-16.63 to -83.36
PLP2	-25.14 to -116.49	-16.98 to -88.4
Jain	-0.84 to 6.53	-0.99 to 4.11
PMF	-15.32 to 47.15	-8.91 to 48.08
DockScore	0.097 to 53.301	0.283 to 47.82

The scores were in higher range in site 1 than site 2. Of the scoring functions used, the PLP and PMF are denoted as negative values. Higher the negative values, stronger are the interactions. The ligands that score well in both the sites were checked for the amino acid interactions in the sites. The natural substrate, 2-ADP Ribose formed hydrogen bonds with D10, I11 and R144 through the adenosine moiety, with V33, S110, G112,

V113 and Y114 through phosphate groups and with D31, N24 and T111 through ribose sugar. There were also non- bonded interactions with other prominent residues around the binding pocket. The ligands that are destined for the inhibition of this receptor activity should interact with these crucial residues strongly so that the binding will not happen with the substrate. Eventually, the strong binding of the phytochemicals with the receptor will result in the loss of the function of macro domain. The phytochemicals that score high values were analyzed for their bonded and non- bonded interactions. These ligands showed favorable interactions with the active site residues. It was noteworthy that the good scoring phytochemicals bind to the conserved aspartic acid (D10).Some phytochemicals used hydrogen bond to interact with D10 while others had non- bonded interactions. This single aspartic acid, conserved through all macro domains is responsible for the specific binding of the adenine base (Malet *et al.*, 2009).The affinity between the receptor and the substrate may get disturbed due to this strong binding. The DockScore of the ligands showed that the receptor- ligand interaction energy and the ligand internal energy were in the most favorable range. The more negative PLP and PMF values indicated the strong binding affinities between the ligands and the macro domain. The Ligscores of the ligands clearly indicated the valid contribution of polar and buried surface area to the receptor-ligand affinity. The interaction between the macro domain and the phytochemicals is directly proportional to the Buried Surface Area. The lipophilic interactions also played a vital role in the ligand affinity towards the macro domain. The Jain scores stood as an evidence for the favorable lipophilic and polar interactions, protein and ligand solvation and entropy of the ligand. The phytochemicals like Quercetin, Fisetin, Taxifolin, Dihydrofisetin, Rhein, Aloe- Emodin and Galangin had scored pretty well in site 1 (**Fig 1**). These scores denote the affinity of these ligands towards the macro domain. Ellagic acid, Taxifolin, Galangin, Ascorbic acid, Pelargonidin, D- Glucosamine, Rhein and Kaempferol scored better in all scoring systems with respect to the second binding site (**Fig 2**). This reflects the binding affinity of the ligands with the target.

All these ligands possess one or more aromatic ring structures that were common with the control ligand. The important physico- chemical parameters like Molecular Weight (MW), Partition coefficient (LogP), Hydrogen Bond Donor and Acceptor (HBD, HBA) were determined using QikProp (**Table 2**). They were in the favorable range as prescribed by Lipinski's Rule of Five (Lipinski, 2004). The drug- like properties of the ligands was thus assured.

Table 2: Important drug- like properties of the screened best scoring ligands.

Ligands	MW	LogP	HBD	HBA
➢ Quercetin	302	1.5	5	7
➢ Fisetin	286	2	4	6
➢ Taxifolin	304	1.5	5	7
➢ Dihydrofisetin	288	1.3	4	6
➢ Rhein	284	2.2	3	6
➢ Aloe- Emodin	270	1.8	3	5
➢ Galangin	270	2.3	3	5
➢ Ellagic Acid	302	1.1	4	8
➢ Ascorbic acid	176	-1.6	4	6
➢ Pelargonidin	271	4	4	1
➢ D-Glucosamine	179	-2.8	5	6
➢ Kaempferol	286	1.9	4	6

The docking results also showed the top leading phytochemicals binding to the amino acids through bonded and non- bonded interactions that were preferred by the control ligand. Aromatic purine ring of adenine present in the control ligand bound to V33 using pi bond which was also attached to the phosphate group of the control ligand. From the docking, it was observed that the top scorers bound to V33 using pi and hydrogen bonds. These binding may allow the phytochemicals to be accepted by the macro domain than the control ligand. Through bonded and non- bonded interactions, these top- scoring ligands bound strongly to the macro domain than the substrate.

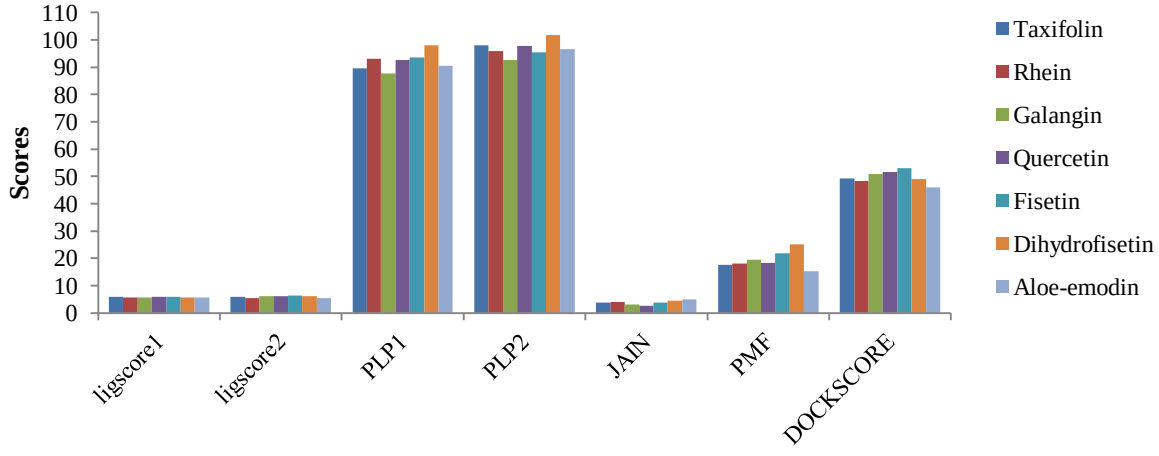

Fig. 1: Distribution of scores of the top scoring ligands binding to Site 1.

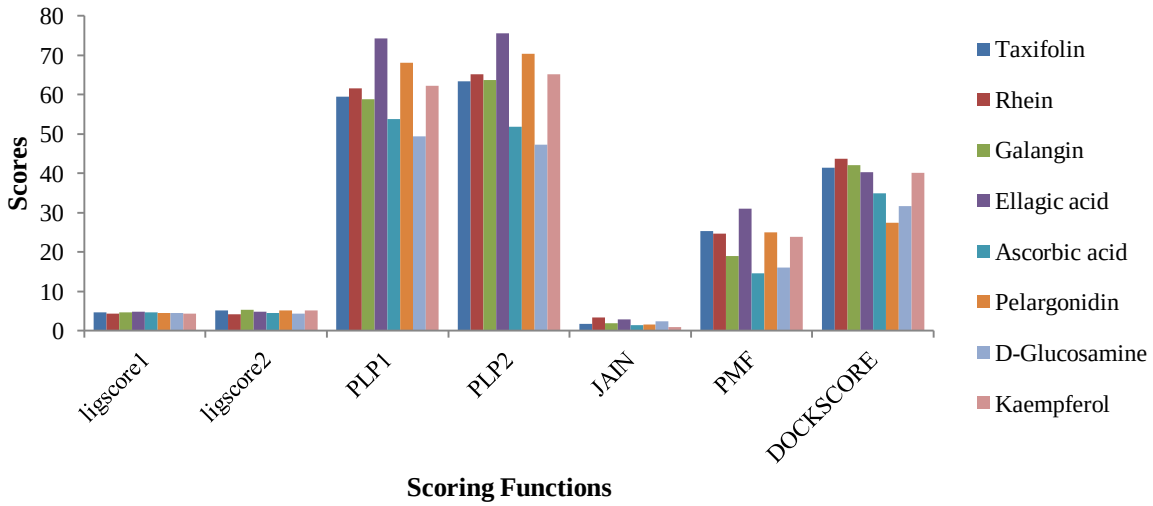

Fig. 2: Distribution of scores of the top scoring ligands binding to Site 2.

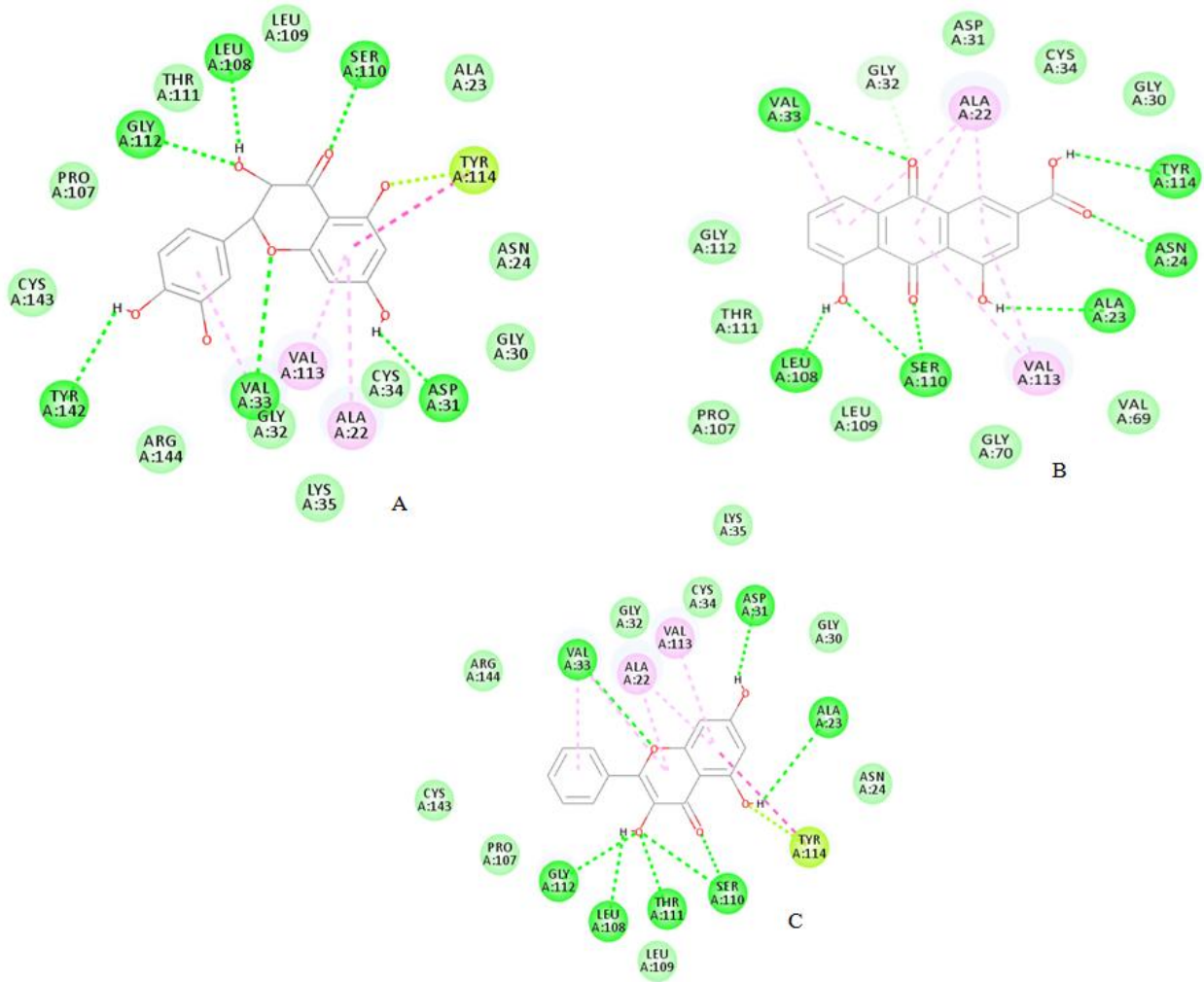

Fig. 3: Interacting amino acids with Taxifolin (A), Rhein (B) and Galangin (C) in Site 1. (Dark green dashes- Conventional Hydrogen Bond, Light green dashes- van der Waals,Purple dashes- pi- sigma, Pink dashes- pi- alkyl, Red dashes- donor- donor).

Taxifolin, Galangin and Rhein bound to both the binding sites 1 and 2 which are near to each other (**Fig 3, 4**). Rhein formed a hydrogen bond with conserved D10 through its hydrogen atom attached to the hydroxyl group. Other two phytochemicals exhibited van der Waals interaction with D10.Hence, their binding to the target can be considered as stronger than other ligands. The scores of the top scoring ligands were higher than the control. The DockScore involves the summation of van der Waals and electrostatic interaction of the protein- ligand complex. The DockScore of 2-ADP Ribose (10.3) was much lower than the other ligands which signify the preference of the phytochemicals to the binding site then the ribose. Thus, these three ligands having satisfied all the necessary parameters may compete with the natural substrate leading to the failure of this protein function.

Taxifolin and Galangin are flavonol, a type of flavonoid. The *in vitro* studies also showed that taxifolin enhanced the efficacy of conventional antibiotics like levofloxacin and ceftazidime (An *et al.*, 2011).Galangin is also known to possess anti- viral properties against herpes simplex, coxsackie, adeno and reo viruses (Meyer *et al.*, 1997). Rhein, also known as cassic acid, is a substance in the anthraquinone group obtained from rhubarb. It is known to possess antibacterial properties (Yu *et al.*, 2008). The internal energies of the ligands were computed using the CharmM force field. The top scoring ligands in both the sites also had favorable internal energies proving that the ligands were comfortably accommodated in the binding sites of the macro domain. Other than these phytochemicals, Ellagic acid scored higher in site 2. This compound also exhibited hydrogen bond with D10 (**Fig 5**). It is a fused four ring compound, present in several fruits such as cranberries, strawberries, raspberries, and pomegranates. It is used as a dietary supplement. It possesses anti-oxidant properties (Seeram *et al.*, 2005). Hence, the literatures also support the pharmacological importance of the screened ligands. The flexibility of the apo form of macro domain was compared to its docked form with the top scoring ligands like Rhein, Taxifolin and Galangin (**Fig 6**). From the data, it can be clearly understood that the flexibility of the amino acids were relatively low for the galangin while compared to the other ligands. This support the fact, that the binding of galangin to the active site did not favor the flexibility of the amino acids residing in the active site.

The binding was such strong that the side chains were made more rigid. Thus, Galangin alone was considered for the molecular dynamics study among the top scoring ligands. The stability of the macro domain and its Galangin bound structure were measured in terms of deviations and fluctuations. The RMSDs were calculated as the function time in the unit of ns (**Fig 7 (a)**). The apo form of macro domain exhibited a peak RMSD of 0.32 nm between 1and 2 ns, whereas the galangin bound form did not have that fluctuation. This clearly indicates the stubbornness of the macro domain structure after binding to galangin. During the course of the simulation, the galangin bound macro domain exhibited a near equilibrium state without any improper fluctuations.

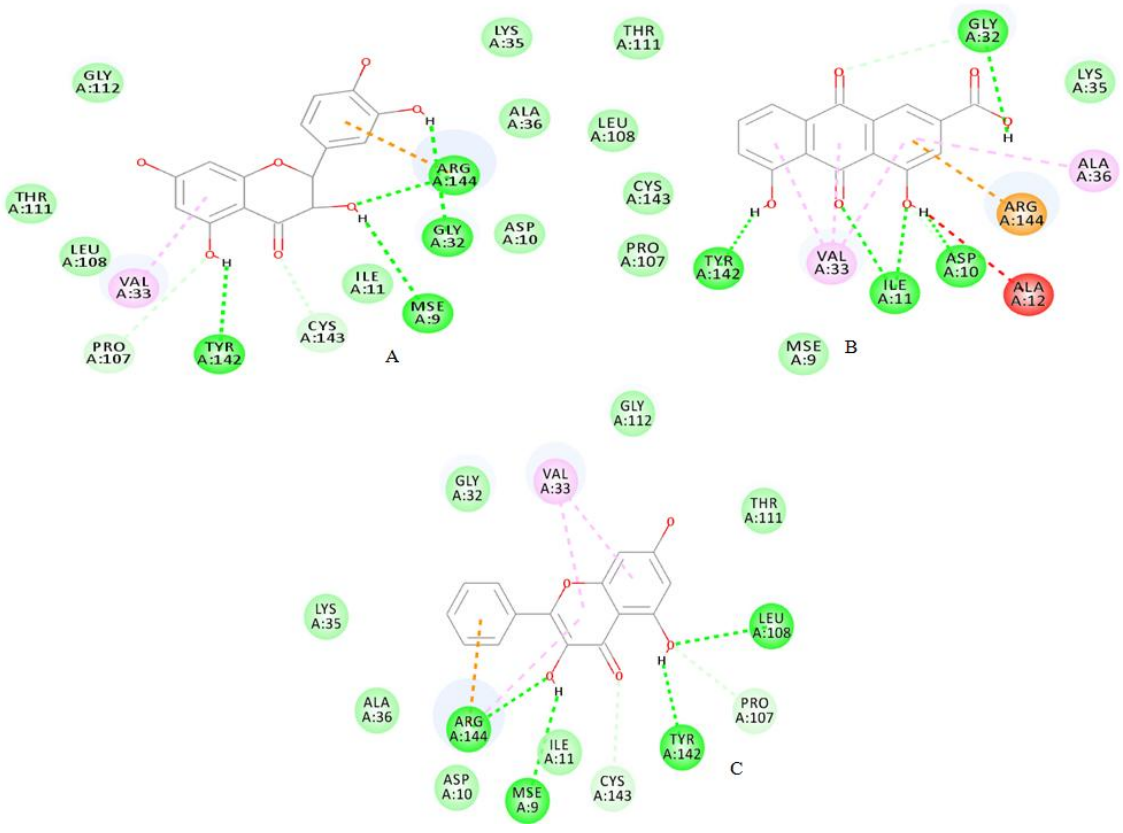

Fig. 4: Interacting amino acids with Taxifolin (A), Rhein (B) and Galangin (C) in Site 2.

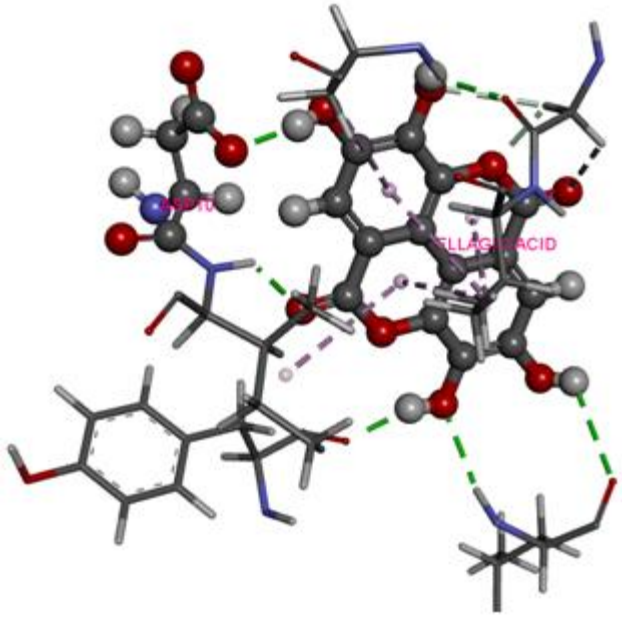

Fig. 5: Ellagic acid making a hydrogen bond (Green dashes) with D10.
Aspartic acid and Ellagic acid are shown in Ball and Stick format. Other interacting residues are in line format.

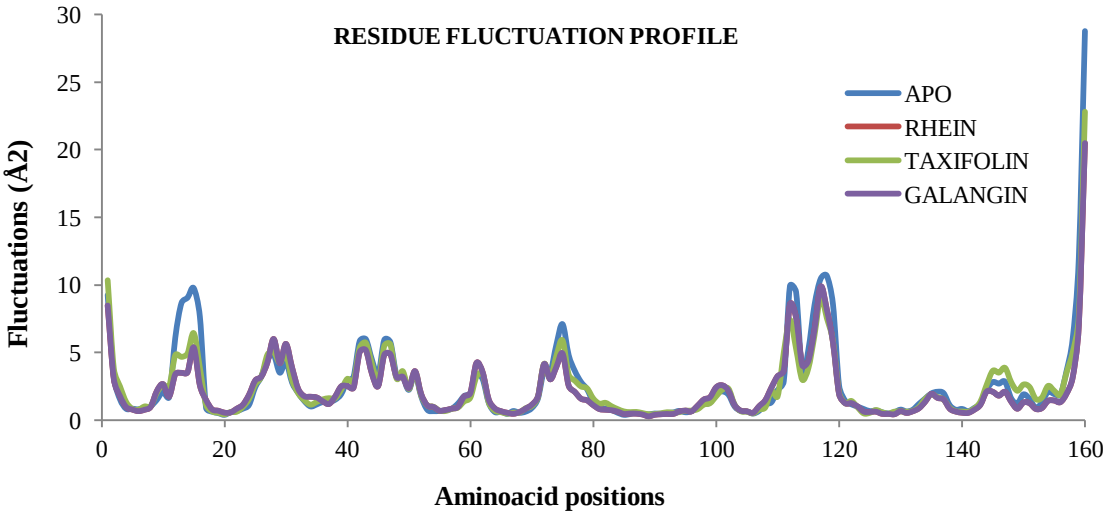

Fig. 6: Amino acid fluctuation profiles of Apo and ligand- bound macro proteins.

This denoted the stability of the ligand bound form. From the graph, it can be seen that the structure of galangin bound macro domain was stabilized after 4ns. When compared to the apo form, the galangin bound form had lower RMSD indicating the stable complex formation with the ligand. The average deviations were 0.225 nm and 0.215nm for the apo and ligand bound forms throughout the period of dynamics. The stability of a protein structure also depends on the flexibility of the local structures. To study this phenomenon, the RMSF plots were drawn for the proteins. From the graph, it can be noted that the fluctuations of most of the amino acids in the ligand bound form were below 0.2nm (**Fig 7 (b)**). Especially the amino acids present in the active site showed a lesser fluctuation after binding to galangin when compared to the apo form. This clearly indicated that the binding of residues to the ligand had restricted its free movement. Since the fluctuations in the ligand bound form were not so drastic, we can conclude that the ligand did not change the overall topology but has affected the local structure comprising the active site. The docking results showed that galangin bound to macro domain by forming hydrogen bonds with the residues. During the simulation period, galangin maintained at least four hydrogen bonds and a maximum of five hydrogen bonds (**Fig 7 (c)**). The retention of hydrogen bonds during the simulation period signified the stability of the protein- ligand complex. This finding was also consistent with RMSD and RMSF data. The R_g for the backbone atoms of apo and galangin bound macro domain was also calculated and plotted against simulation time (**Fig 7 (d)**). The R_g is a measure of the structural changes happening during simulations. This value was lower for the ligand bound form than the apo form in most of the simulation time.

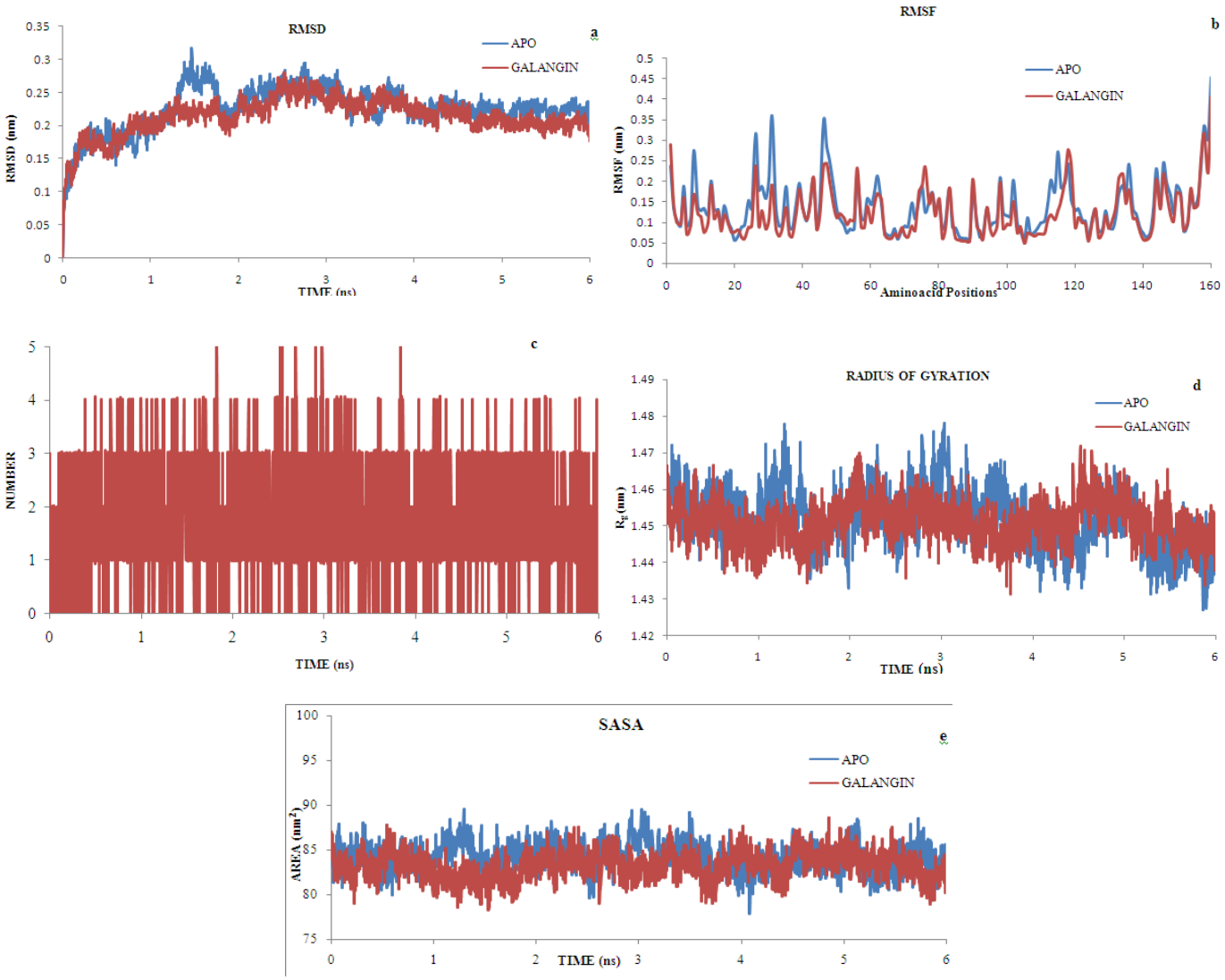

Fig. 7: (a) RMSD of apo and galangin bound macro domain for 6ns simulation. (b) RMSF of backbone atoms of apo and galangin bound macro domain. (c) Hydrogen bond count of Galangin with macro domain. (d) Rg of backbone atoms of apo and galangin bound macro domain. (e) SASA of apo and galangin bound macro domain.

This showed the compactness of the structure after binding to galangin. This was consistent with the other measures of stability like RMSD and RMSF. The overall change in the shape of the macro domain during the simulation was studied using SASA (**Fig 7 (e)**). The SASA of the galangin bound protein was lower than the apo form between the time period of and 2 ns. This reduction in SASA could be due to the inaccessibility of the protein after binding to galangin. This kind of similar pattern was also observed at the end of the simulation period. The molecular dynamics results support the fact that the binding of galangin to macro domain will affect the protein structure and eventually the function. These studies have demonstrated the macro domain of the Chikungunya virus as a prominent drug target and also have revealed the potentials of phytochemicals as leads for anti- viral drug designing.

CONCLUSION

Drug discovery is a huge time and money consuming process. The target identification is a key step in the identification of any new drug. The scope of the computer aided drug designing lies on a proper target. A target of an anti- viral drug should be such that its inhibition should lead to the loss of viral function. Macro domain can be considered as a potential drug target based on its vital functions with respect to chikungunya virulence. Based on the insight from the multiple scoring functions, drug- like properties, amino acid interactions and molecular dynamics, we conclude that the phytochemicals screened have the ability to bind with the macro domain in place of 2- ADP Ribose. These ligands possess more affinity towards the active site of macro domain. The interaction of this kind may inhibit the normal potential functions

of macro domain. Hence, these phytochemicals can be suggested as a cure for the disease with respect to the target considered. The plant sources of these phytochemicals can be considered as a treatment strategy for curing Chikungunya based illness. Using computer aided drug design and chemoinformatics approaches, it is also possible to design novel inhibitors targeting chikungunya virus based on the structural features of the top scoring ligands as suggested by this work.

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