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In silico analysis of 2A protease as a prophylactic and therapeutic target against Hand, Foot, and Mouth disease

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Enteroviruses, particularly EV-71, are responsible for the major periodic outbreaks of Hand, Foot and Mouth disease in children. To mitigate the severity of disease in pediatric populations, developing prophylactic and therapeutic agents is critically important. The present *in silico* study focuses on EV-71 2A protease as the putative target for predicting novel drugs and epitope-based vaccines against HFMD. Various natural compounds, viz. flavonoids, terpenoids, and phenolics, were screened using the H-Dock molecular docking server to analyse their binding affinities with 2A protease. Flavonoids such as protopanaxatriol (-148), Luteolin (-141), and Resibufogenin (-140) were identified as the potent inhibitors of 2A protease, based on their docking scores being comparable to that of known inhibitor CW-33 (-155). Further, the bioinformatics approach was also used to construct a multi-epitope polypeptide that contains T-cell and B-cell epitopes of 2A protease alongside adjuvants and linkers to improve immunogenicity. The designed vaccine construct was further validated based on scores of immunological parameters, viz. allergenicity, antigenicity, solubility and physicochemical properties. Molecular docking studies with immune receptors reveal binding scores of -213 for TLR3, -217 for TLR5, and -233 for TLR8, indicating a promising immune stimulatory response. However, future *in vitro* and animal studies will be essential to establish this research as a benchmark in terms of drug design and vaccine development.

Keywords: 2A protease, EV-71, HFMD, Phytochemicals, Vaccine construct

IPC code; Int. cl. (2021.01)− A61K 36/00

Introduction

Hand, foot, and mouth disease (HFMD) is a common viral illness primarily affecting children under the age of 10 years but can also occasionally affect adults. Fever, vesicular rashes on the hands, feet and buttocks, as well as oral mucosal ulcers, are the major symptoms. HFMD typically can resolve on its own, but its severity may develop life-threatening consequences such as meningitis, encephalitis, acute flaccid paralysis (AFP), and neurorespiratory syndrome. HFMD thus represents a significant hazard to public health[1]. The primary Coxsackie A viruses (CVA 16) and Enterovirus A71 (EV 71) are the human enterovirus species of picornaviruses that cause HFMD. The Picornaviridae family of EV71 and CVA 16 encode a large polyprotein precursor consisting of four structural proteins (VP1, VP2, VP3 and VP4) and seven non-structural proteins (2A pro, 2B, 2C, 3A, 3B, 3C pro and 3D pol) that are crucial for viral RNA replication and translation. Proteases (2A pro and 3C pro) encoded by EV-71 help in the cleavage of polyprotein to capsid (VP0, VP1, VP3) and replication proteins (2A pro -2C and 3A-3D pol)[2]. EV 71- $2A^{pro}$ helps in cleavage of host elongation factor eif4GII, which results in the suppression of viral spread, cellular immune responses and host translation machinery. EV-71 antagonises type I IFN signalling by reducing the level of interferon receptor 1 (IFNAR1)[3]. 2A protease has a greater role in undermining the activities of host cells, such as inhibiting nucleocytoplasmic transport, host protein synthesis, and evading innate immunity[4]. Hence, EV-71 $2A^{Pro}$ could be an effective therapeutic target against EV-71 infections. Till date, CW-33 (ethyl 2-(3′,5′-dimethylanilino)-4-oxo-4,5-dihydrofuran-3-carboxylate), a synthetic derivative of fluoroquinoline alkaloid has been confirmed as a potent inhibitor of EV -71 *in-vitro*[5]. Despite the urgent need, it is unrealistic to develop new drugs within a short period. Traditional drug discovery requires a very long period of investment. Currently, there are no specific antiviral agents that can be used in the treatment of the onset of HFMD. Antiviral drugs such as acyclovir and ribavirin can be used only in severe cases of

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aseptic meningitis, cardiopulmonary complications and cardiorespiratory failure among HFMD patients[6]. However, long-term exposure to these antivirals may lead to deterioration of immune response and significantly cause neuropsychiatric side effects[7]. Thus, phytochemicals, being natural compounds, can be used as alternative therapeutics for the treatment of HFMD. Screening plant compounds using *in silico* techniques such as molecular docking provides a fast and effective strategy to identify inhibitory compounds against a viral protein based on binding affinities in comparison to traditional drug discovery methods[8]. Vaccination is always considered to be an emergent preventive approach for any infectious viral disease. Vaccine development is a time-consuming and costly process that involves isolation, cultivation and identification of the antigenic components from pathogenic microorganisms[9]. Computational approaches using bioinformatics methods help to screen a number of novel and putative vaccine targets from genome sequence studies, thereby accelerating the process of vaccine development[10]. Thus, *in silico* techniques can be a pioneering approach used during all the stages of the discovery and development of vaccines. These studies might encourage the choice of robust screening of antigen or epitope, adjuvants (such as liposomes and nanoparticles), doses and dosing schedules to enhance the future *in vitro* research in the design of vaccines. Till now, there is no US-FDA approved vaccine against EV-71 infections. Three whole-cell inactivated vaccines against EV-71 using C4, B4 and B5 genogroups, respectively, have been authorized by the China National Medical Products Administration (NMPA) but failed in vaccine effectiveness and cross-protection against different strains of EV-71[11]. The success of protease inhibitors in treating other viral infections, such as HIV and Hepatitis C, supports the feasibility and effectiveness of similar strategies against EV71[12,13]. Targeting 2A proteases of EV- 71 offers a promising strategy for vaccine development due to their crucial role in viral replication and the elicitation of strong immune responses. Thus, the present *in silico* study was designed with the aim of predicting novel phyto-based drugs and epitope-based vaccine constructs against EV 71 using 2A protease.

Materials and Methods

Sequence retrieval

The protein sequence of EV -71 $2A^{Pro}$ (accession no. AFL71132) consisting of 2193 amino acids was retrieved in FASTA format from the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) to predict suitable epitopes for making vaccine against HFMD.

Physico-chemical parameters

All physico-chemical parameters of EV -71 $2A^{Pro}$, viz. molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity were evaluated by the ProtParam tool of Expasy. These analyses are essential to check the behaviour of proteins in the biological system (http://us.expasy.org/protparam. html).

Structure prediction, validation and refinement

The GOR IV tool was used to predict the secondary structure of EV-712A^{Pro}. This is an *ab initio*-based method that predicts the structure based on the relative propensity of each amino acid belonging to a certain secondary structure element. The highest-scored state defines the conformational state for the centre residue (https://npsa-prabi.ibcp.fr/cgi). The tertiary structure was built using phyre2 server, which is an advanced homology-based detection method to predict 3-D models using Hidden Markov Models with % confidence modelling (http://www.sbg.bio.ic.ac.uk/phyre2/)[14]. Further, structural refinement was done by Galaxy refinement from Galaxy Web Server molecular (https:// galaxy.seoklab) that refines loop or terminus regions of protein structure by *ab initio* modelling with the scoring of GDT-HA less than 1 and MOLPROBITY in the range of 1-3[15]. The predicted model was further validated by the SAVESv6.0 database PROCHECK (https://saves.mbi.ucla.edu/) and the ProSA web tool (https://prosa.services. came.sbg.ac.) PROCHECK validates the stereo-chemistry of the protein structure by analysing residue by residue geometries generated by the Ramachandran plot. Plot showing a higher percentage of residues in core regions represent good quality structure[16]. ProSA utilises the structure's energy to compute distance-based pair potentials of mean force for validation. These energies are used to derive a Z-score ($\leq$10) and a plot as an indicator of model quality and residual energies, respectively[17].

Structures of natural compounds and immune receptors

3-D Structure of various natural compounds and Immune receptors (TLR 3,5,8) used in this study were downloaded from NCBI Pub chem in SDF format and

then converted into PDB format using Open Babel Software (http://openbabel.org).

B cell and T cell epitope prediction

The prediction of B cell epitope was made by using the BepiPred Linear epitope prediction tool that predicts B cell epitopes based on the propensity of amino acids to predict regions of proteins that are likely to be antigenic or immunogenic[18] (https:// www.iedb.org). T cell epitope was predicted using MHC I and MHC II prediction tools (www.iedb.org) based on Artificial neural networking and scoring matrices derived from combinatorial peptide libraries and selecting alleles occurring in at least 1% of the human population. The IEDB recommends making selections based on a consensus percentile rank of the top 20%, which captures 50% of the immune response[19]. The peptide binders to MHC I and MHCII were selected using the Rankprep server, which predicts the peptides based on position-specific scoring matrices (https://imed.med.ucs.es).

Antigenicity, allergenicity and toxicity prediction

The highest score predicted peptide sequence was chosen from both the B cell and T cell predicted epitopes list. Antigenicity was checked by the Vaxijen2.0 server (http://www.ddg-pharmfac.net/ vaxijen/) that classifies antigens based on auto | cross-covariance (ACC) transformation of protein sequences into uniform vectors of principal amino acid properties with the threshold value of 0.4. Allergenicity was checked by Aller TOP 2.0 version online tool (http://www.ddgpharmfac.net/AllerTOP/) that predicts recombinant protein allergenicity on auto cross-covariance by determining it to k nearest neighbour algorithm. Toxigenicity was checked using a Toxipred server (http://www.ddg-pharmfac.net/ Toxipred) that uses support vector machines (SVMs) as supervised learning models to calculate scoring matrices with a threshold value of 0.5.

Prediction of epitope-based vaccine construct

The highly antigenic, immunogenic, non-toxic, and non-allergenic epitopes were selected for the final vaccine construct. 50S ribosomal protein L7/L12 was added as an adjuvant to enhance the efficacy, immunostimulatory properties, life span and safety profile of peptide-based vaccines[20]. It was linked via the EAAAK linker at the N terminal of the vaccine to improve expression and bioactivity. The B cell and HTL were linked via the GPGPG linker to stimulate TH lymphocyte (HTL) responses, and the CTL epitope was linked via the AAY linker to improve the expression of the target proteins and immunogenicity of the multi-epitope vaccine[20]. Further, the C terminal end was linked with the HHHHHH (6HIS) linker as it may help to purify recombinant protein for future *in vitro* studies[21]. The predicted final vaccine construct was further submitted to VaxiJen, Aller TOP, and Toxipred servers to evaluate its immunogenic response.

Structure prediction, refinement and validation of vaccine construct

All the physico-chemical parameters of the vaccine construct were analysed using Expasy's ProtParam tool. The secondary structure of the vaccine construct was predicted using PSI protocol based on artificial neural network machine learning methods. The tertiary structure of the epitope was built using the phyre2 server. Galaxy Web Server was used to refine the structure of the predicted epitope. The model was further validated by the SAVESv6.0 database, including PROCheck and ProSA web tools.

Molecular docking

The predicted target EV - 71 $2A^{pro}$ was docked with substrates (IFNα/β & eIF4E), inhibitor (CW- 33) and phytochemical compounds. Similarly, the vaccine construct of EV - 71 $2A^{pro}$ was docked with immune receptors viz. TLR 3,5,8 by using an H-DOCK server (https:// hdock.phy.hust.edu.cn/.), which predicts the interaction interface of ligand and receptor by calculating binding affinities using template-based modelling. Ten models are predicted with docking scores ranging from highest to lowest value of binding affinity with query coverage of 1.00%. The scoring function of this tool is based on energy calculations, shape complementarity and desolvation energies for protein-protein interactions.

Results

Bioinformatic analysis

The retrieved sequence of EV 71 $2A^{pro}$ contained linear DNA of 2193 bp. The highest significant hit revealed by BLASTp analysis (E value = 0) confirmed the successful retrieval of the sequence used in the study as it showed 99.9% sequence identity with Polyprotein of Enterovirus A-71 (accession number AEQ61271.1) with 100% query coverage. The various physicochemical parameters of EV - 71 $2A^{pro}$ were evaluated using the Expasy Protparam tool (Supplementary Table 1).

Structure prediction, refinement and validation

Secondary structure of EV -71 $2A^{Pro}$ was predicted using GOR IV tool having 50 % random coil, 28% alpha helix, 20% extended strand (Supplementary Fig. 1). Tertiary Structure was predicted by phyre2 server based on template and model similarities. The template identified for EV -71 $2A^{Pro}$ was coding for RNA dependent polymerase structure of EV-71 in complex with GTP (template id c3n6mA) having sequence identity of 97% with 100% confidence modelling score (Supplementary Fig. 2). The structure was further refined by GALAXY refine server. The refinement of the crude model predicted five structures models, out of which Model 1 was chosen for further prediction based on GDT-HA= 0.9 and MOLPROBITY = 1.5. Further structure was validated by the SAVES 6.0 PROCHECK webserver and ProSA web tool (Fig. 1a,b). PROCHECK provided an overall summary of the Ramachandran plot with 91% residues in favoured core regions representing good quality structure. ProSA web tool gave an overall Z score of -10.43 for a predicted model of EV -71 $2A^{Pro,}$ indicating that the model falls within the scoring range commonly observed in similar size-native proteins.

Immunoinformatic analysis of vaccine construct

The vaccine design construct consists of 462 amino acids from selected peptides of B cell epitope, MHC I and MHC II epitope based on high binding affinity score single peptide from each type of epitope. The IEDB server was used to predict the immune epitopes used in the vaccine construct. The predicted T cell and B cell epitopes were 66 and 75% identical with the genome polyprotein of coxsackievirus A. The prediction is based on the sequence similarity with the highest antigenicity, matching it with corresponding amino acids with the sequences available in the database using the HMM approach. The peptide binders to MHCI and MHCII molecules from protein sequences were predicted using the RANKPEP server. The selected peptide was 9 mer, which was ranked after their binding to H2-Db 9 mer. Furthermore, linkers and adjuvants were added to the N and C terminals of the vaccine construct to increase its immunogenicity. Vaccine construct peptide on further submission to Vaxigen, Allergen, and Toxinpred server passed all the immunogenic parameters (Table 1 and Table 2). The physico-chemical parameters of the vaccine construct were evaluated by protparam tool (Supplementary Table 2).

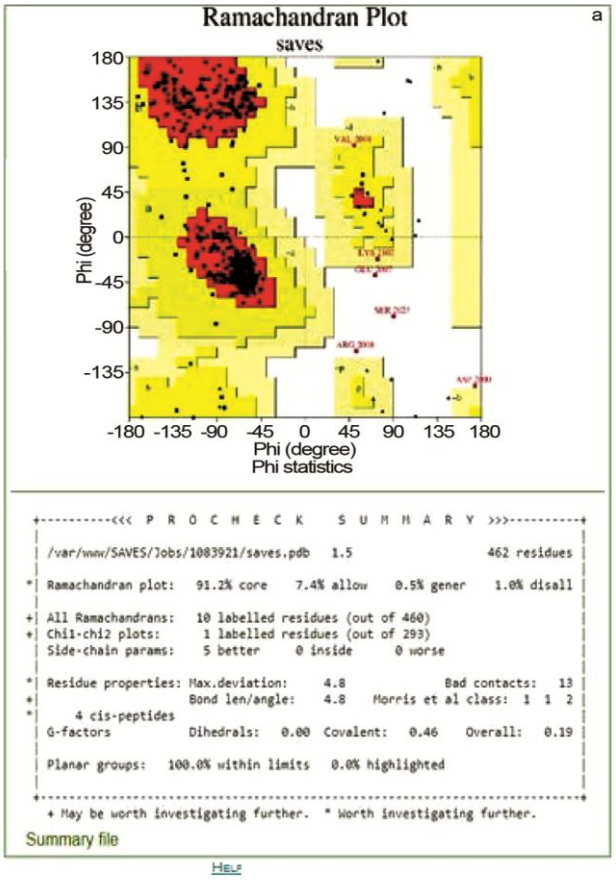

Fig. 1 — a) Summary file of PRO CHECK indicating all statistical and geometrical parameters of Ramachandran plot; and b**)** ProSA web modelled structure evaluation indicating Z- score of -10.43 highlighting the protein in NMR and X-ray crystallographic region.

Table 1 — List of selected peptides and their immunogenic parameters

Immunogenic Parameters	B cell peptide	MHC class I Peptide	MHC class II Peptide
Sequence	VSTQRSGSHENSA	NTAYIIALA	TAYIIALAAAQKNFT
Antigenicity	0.4957 Antigen	0.4957 Antigen	0.4957 Antigen
Allergenicity	Non- allergen	Non- allergen	Non- allergen
Toxicity	-1.01 Non-toxic	-0.71 Non-toxic	-1.08 Non-toxic
Protein solubility	0.67	2.17	0.01

Table 2 — List of final vaccine construct peptide and its evaluated immunogenic parameters

Sequence	EAAKVSTQRSGSHENSNSACPGPGNTAYIIA LAAYTAYIIALAAAQKNFHHHHHH
Antigenicity	0.495 score indicating probable antigen
Allergenicity	Non- allergen
Toxicity	-1.13 Non-toxic

Structure prediction, refinement and validation of vaccine construct.

The secondary structure of the vaccine was predicted using the PSIPRED tool (Supplementary Fig. 3), revealing the presence of helices and coils. The tertiary structure was predicted by the phyre2 server using c1dgj4 template id coding for poliovirus fragments vp1, vp2, vp3, and vp4 with a sequence identity of 56 with 90% confidence modelling score (Supplementary Fig. 4). The structure was refined by GALAXY refine server. The refinement of the crude model predicted five structured models, out of which Model 1 was chosen for further studies with scores of GDT-HA=0.81 and MOLPROBITY=3.0.

The validation of the 3-D structure was done by the SAVES 6.0 PROCHECK database, which provided an overall summary of the Ramachandran plot with 84% residues in favoured core regions representing moderate quality structure. ProSA web gave the Z-score value of -2.2 for the designed vaccine construct, indicating that the modelled protein lies within the range of similar size-native proteins (Fig. 2a,b).

Molecular Docking

Evaluation of various substrates, plant compounds, and immune receptors with respect to the binding affinities of ligand and receptor showed that each compound gave a scoring result that was compatible with that of target 2A protease. Out of the top ten predicted models, the highest scoring value at the most negative intermolecular binding energy is considered favourable for showing the strongest binding affinity. Molecular docking of EV-71 $2A^{Pro}$ with IFN α/β and eif4E predicted score values of (-267) and (-242), respectively. Inhibitor (CW -33), a synthetic derivative of fluoroquinolones alkaloid,

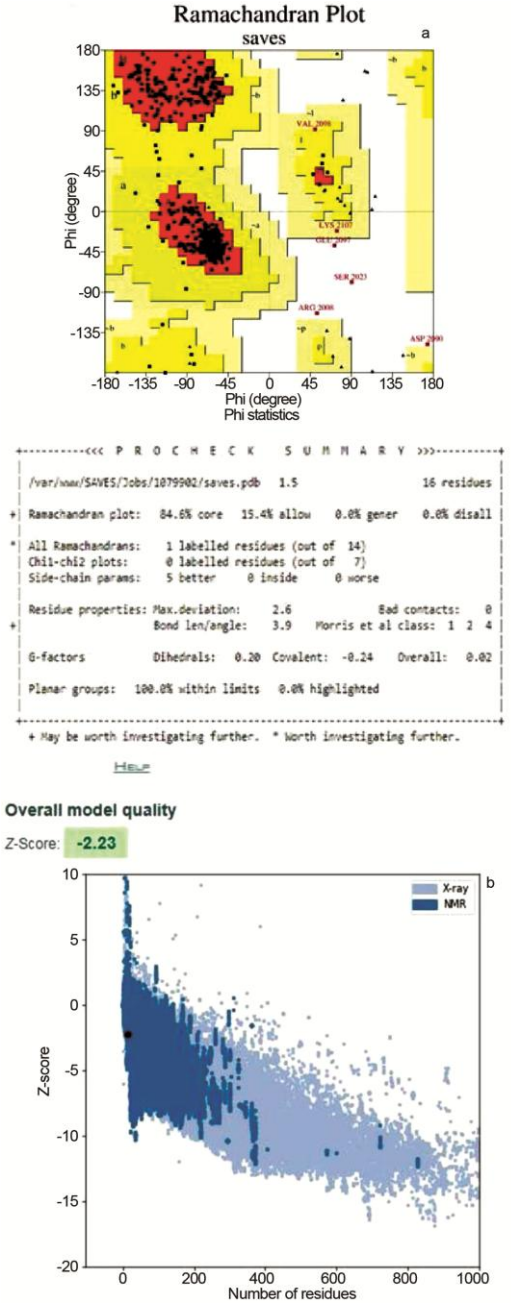

Fig. 2 — a) Summary of all statistical and geometrical parameters of vaccine construct by Ramachandran plot; and b) ProSA web modelled structure evaluation of vaccine construct indicating Z- score of -2.23 highlighting the protein in NMR and X-ray crystallographic region.

showed a score value of (-155). Natural plant compounds such as Protopanaxatriol (-148), flavone Luteolin (-141), Resibufogenin (-140) showed score in the range of inhibitor CW-33 and higher binding affinity with target protein of 2A protease as compared to antiviral drugs such as Acyclovir (-121), oseltamivir (-118), Ribavirin (-116) (Table 3). Further docking of predicted immune receptors with known epitope-based vaccine construct sowed a good score value viz. TLR 3(-213), TLR 5 (-217) and TLR 8 (-233), which were in the range of *in vivo* substrates viz.IFN α/β and eif4E indicating strong binding affinities and suggesting it to be a potent immunogen (Table 4) (Fig. 3).

Table 3 — Binding affinity scores of target protein 2A protease with plant compounds, inhibitors, substrates and anti-viral drugs calculated by H Dock server

Compound	Class/Mode of action	Source	Score
IFN alpha/beta (substrate)	Immune response mediator produced in the leukocytes infected with virus, inhibition of interferon-stimulated genes (ISG)	rcsb.org PDB ID 5XBV	-267
eif4E (Substrate)	Translation initiation factor recognises and binds the 7-methylguanosine containing mRNA cap during initiation of protein synthesis in EV 71	rcsb.org PDB ID 3S98	-242
CW -33 (Inhibitor)	Synthetic derivative of fluoroquinolones alkaloid, inhibits EV – 71 replication by binding to its active site	Pubchem id 132574745	-155
Ampelopsin	Anti-inflammatory properties	*Ampelopsis grossendetata* plant extract	-155
Penduletin	Flavonoid, anti-inflammatory properties	*Psidia viscosa* plant	-149
Protopanaxatriol	Tetra terpenoid, inhibit 3C protease activity of coxsackievirus B3	*Gynostemma pentaphyllum*	-148
Asiatic acid	Tri terpene anti-inflammatory properties	*Centella asiatica*	-145
Luteolin	Tetra hydroxyl flavonoid, Inhibit RNA synthesis of EV -71	*Verbascum lychnitis*	-141
Resibufogenin	Diterpene flavonoid, Disrupt protein synthesis of EV -71	*Sclerophyrus mauritanica*	-140
Ferulic acid	Phenolic compound, anti- inflammatory, apoptosis inhibitor in SARS CoV	*Halophyllum griffithianum*	-139
Aloe emodin	Anthraquinone inhibit viral replication by IFN signaling response in EV -71	*Rhamnus davurica*	-139
Scutellarin	Glycosyloxyflavone, Proteasome inhibitor in EV 71	*Scutellaria barbata*	-138
Corilagin	Ellagitannin antiviral down regulator of 3 C protease EV-71	*Euphorbia fischeriana*	-136
Apigenin	Trihydroxy flavonoid , disrupts viral replication of EV-71	*Verbascum lychnitis*	-132
Lycorine	Alkaloid, protein synthesis inhibitor of EV 71 and CVA 16	*Sternbergia clusiana*	-131
Formononetin	Glycoside, reduce RNA and protein synthesis in EV 71	*Pterocarpus indicus*	-129
Gemcitabine	Nucleoside metabolic inhibitor antiviral drug against EV 71	Antimetabolite drug Pubchem id 60750	-127
Tartaric acid	Hydroxycarboxylic acid, inhibits viral replication of RNA viruses	Found in fruits	-125
Acyclovir Antiviral drug	Inhibits viral DNA polymerase activity of EV 71 and other DNA viruses	Synthetic analog of purine nucleoside, guanosine	-121
7 –hydroxy isoflavone	Inhibits protein synthesis of EV 71 at early stage	*Muntingia calabura*	-120
Raoulic acid	Anti-viral properties against EV 71	*Rauolia austrails*	-120
Ribavirin	Anti-viral drug Viral polymerase inhibitor of EV 71	Synthetic nucleoside antimetabolite analogue	-118
Oseltamivir	Anti-viral drug Neuraminidase inhibitor acts as adjuvant in inhibition of EV 71	Synthetic amino acid ester belong to acetamides	-116
Caprylic acid	Anti-viral activity against wide range of enveloped virus	Found in mammal milk	-115
Caffeic acid	Anti-viral cytotoxic activity against RNA and DNA viruses	*Pavetta indica*	-101

Table 4 — Binding affinity scores of epitope vaccine construct with TLR (Immune receptors) calculated using H – Dock Server

Human Immune Receptors	Source and Function	Reference (rcsb.org)	Score
TLR 3	Initiates downstream signal transduction, for the up-regulation of IFN-α/β expression and induction antiviral protein (AVP) synthesis activity	PDB ID 3J0A	-213
TLR 5	Works on cell surfaces like epithelium cells and monocytes in humans, that provides protective innate immunity	PDB ID 5GS0	-217
TLR 8	Helps in the recognition of viral and bacterial pathogens.	PDB ID 3w3g	-233
IFN alpha/beta (substrate)	Suppression of interfere on stimulated genes by EV -71	PDB ID 5XBV	-267
eif4E (Substrate)	Inhibition of mRNA cap formation by binding to 7 –methyl guanosine during initiation of protein synthesis in EV 71	PDB ID 3S98	-242

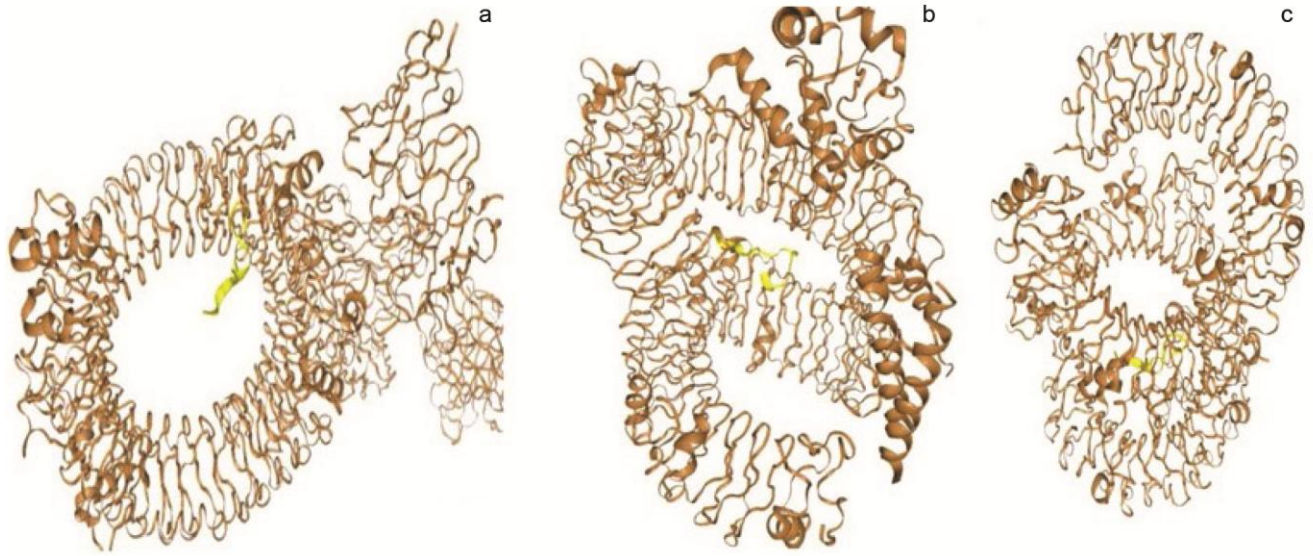

Fig. 3 — Docking model of human Toll-like receptor, a) TLR 3; b) TLR 5; and c) TLR 8 with vaccine construct. The toll-like receptor is shown in brown, and the vaccine construct interaction is shown in yellow.

Discussion

Hand, Foot, and Mouth Disease is a major disease amongst children that is spread by oral ingestion, passes from the gastrointestinal tract or upper respiratory tract to regional lymph nodes and may further spread to multiple organs. Its severity brings along several conditions of aseptic meningitis, polio-like syndrome and clinical manifestation of CNS[22]. As per previous data, 90% of cases were diagnosed in children below the age of 5 years. The mortality rate of this disease was found to be 84.02% amongst children below the age of two years[23].

With the emergence of finding a prominent and effective alternative to fight against HFMD, 2A protease of EV 71 always remained a less evaluated component with only two known weak inhibitors (LVLQTM Peptide and CW-33) to date[24]. LVLQTM peptide is an inhibitor of 2A protease and works by inhibiting the cleavage of eIF4G, which is a eukaryotic initiation factor essential for translation[25]. CW-33 is a strong inhibitor whose mechanism depends on the induction of cell apoptosis by binding to the active site of EV-712A^{pro4}. 2A protease has already been used as a target for the combined cleavage of eIF4G1 and eIF4G2 that led to a shutdown of cap-dependent translation using dual *Renilla* and firefly luciferase mammalian expression construct in Rhinovirus to suppress viral infection[26]. Proteases like M^{Pro}, 3CLPro, and Papain have also been considered as potential drug targets and vaccine targets against SARS-CoV infection[27,28]. Molecular docking strategies for identifying potential drug targets against EV 71 have been previously studied using 3C protease, which was further confirmed to be inhibitory using *in vitro* and i*n vivo* studies[29]. In our study, flavonoids such as Luteolin, Resibufogenin, Penduletin and Apigenin were docked and showed good binding affinity with EV -71 2A^{Pro} in comparison to the synthetic drugs such as acyclovir and ribavirin used in severe cases to treat HFMD[30,31].

A similar report of inhibition of viral replication by Luteolin was observed in EV 71/CVA 16[32]. Comparable results were found using Resibufogenin against EV -71 infection *in vitro*[33]. Similarly, Penduletin inhibits RNA replication in EV 71, and Apigenin has been reported as a potent anti-viral agent to inhibit the IRES-dependent activity of EV 71[34,35]. These flavonoids have also been found effective against SARS-CoV-2 virus using 3CL protease as target[30].

On evaluating the binding affinities of the vaccine construct with immune receptors TLR 3,5,8, it was observed that they exhibited a good affinity with that 2A protease vaccine construct. Epitope-based vaccines were also designed to fight Ebola virus by using VP30, VP24, VP40 GP, and NP proteins as targets[36]. Similarly, cell surface binding proteins were predicted as targets against monkeypox virus using similar computational approaches[37]. A new multi-epitope vaccine candidate has been constructed against the Chikungunya virus using structural proteins viz.E1, E2, 6 K, and E3 via immunoinformatics and immune simulation analysis approach and further visualising its expression in *E. coli* using *in silico* cloning[38]. Thus, predicted plant compounds and epitope-based vaccine targets in our study can work efficiently for further *in vitro* and *in vivo* experiments in terms of practical implications for successful treatment against HFMD.

Conclusion

Phytochemicals have been a significant focus of research in identifying anti-viral compounds and offering an effective alternative to synthetic drugs. Our present study has identified natural compounds, such as Protopanaxatriol, Luteolin, and Resibufogenin, to be putative drug candidates against target 2A proteases using molecular docking studies. However, further *in vitro* validations, such as biochemical and cell-based assays, are mandatory for assessing the anti-viral activities of these compounds against HFMD infections. Extensive immunoinformatic analysis identified 2A protease as a highly recommended target for constructing a competitive epitope-based vaccine against EV-71. The vaccine construct passed all immunogenic parameters in terms of prediction of T-cell and B-cell epitopes, MHC peptide and HLA predictions, antigenicity, toxigenicity and allergenicity. Also, molecular docking studies showed a high affinity of the proposed vaccine construct with immune receptors (TLR 3, 5 and 8), indicating the ability to elicit both humoral and cellular immune responses. However, further *in vitro* and animal model experiments are required to verify the predictions of the designed vaccine construct. Thus, the implications of 2A proteases as the prophylactic and therapeutic target for reducing HFMD infections will, in turn, improve public health outcomes.

Conflict of interest

The authors declare no conflict of interest.

References

1 Lu G, Qi J, Chen Z, Xu X, Gao F, *et al.*, Enterovirus 71 and coxsackievirus A16 3C proteases: binding to rupintrivir and their substrates and anti-hand, foot, and mouth disease virus drug design, *J Virol*, 2011, **85**(19), 10319-10331, doi: 10.1128/JVI.00787-11.

2 Baggen J, Thibaut H J, Strating J R P M and van Kuppeveld F J M, The life cycle of non-polio enteroviruses and how to target it, *Nat Rev Microbiol*, 2018, **16**(6), 368-381, doi: 10.1038/s41579-018-0005-4.

3 Lu J, Yi L, Zhao J, Yu J, Chen Y, *et al.*, Enterovirus 71 disrupts interferon signaling by reducing the level of interferon receptor 1, *J Virol*, 2012, **86**(7), 3767-3776, doi: 10.1128/JVI.06687-11.

4 Wei J, Lv L, Wang T, Gu W, Luo Y, *et al.*, Recent progress in innate immune responses to Enterovirus A71 and viral evasion strategies, *Int J Mol Sci*, 2024, **25**(11), 5688, doi: 10.3390/ijms25115688.

5 Wang C Y, Huang A C, Hour M J, Huang S H, Kung S H, *et al.*, Antiviral potential of a novel compound CW-33 against Enterovirus A71 via inhibition of viral 2A protease, *Viruses*, 2015, **7**(6), 3155-3171, doi: 10.3390/v7062764.

6 Xing W, Liao Q, Viboud C, Zhang J, Sun J, *et al.*, Hand, foot, and mouth disease in China, 2008-12: an epidemiological study, *Lancet Infect Dis*, 2014, **14**(4), 308-318, doi: 10.1016/S1473-3099(13)70342-6.

7 Zareifopoulos N, Lagadinou M, Karela A, Kyriakopoulou O and Velissaris D, Neuropsychiatric effects of antiviral drugs, *Cureus*, 2020, **12**(8), e9536, doi: 10.7759/cureus.9536.

8 Zhao Y, Tian Y, Pan C, Liang A, Zhang W, *et al.*, Target-Based *In silico* screening for phytoactive compounds targeting SARS-CoV-2, *Interdiscip Sci*, 2022, **14**(1), 64-79, doi: 10.1007/s12539-021-00461-4.

9 Bidmos F A, Siris S, Gladstone C A and Langford P R, Bacterial vaccine antigen discovery in the reverse vaccinology 2.0 Era: Progress and challenges, *Front Immunol*, 2018, **8**(9), 2315, doi: 10.3389/fimmu.2018.02315.

10 Delany I, Rappuoli R and Seib K L, Vaccines, reverse vaccinology, and bacterial pathogenesis, *Cold Spring Harb Perspect Med*, 2013, **3**(5), 012476, doi: 10.1101/cshperspect.a012476.

11 Chang Y K, Chen K H and Chen K T, Hand, foot and mouth disease and herpangina caused by enterovirus A71 infections: A review of enterovirus A71 molecular epidemiology, pathogenesis, and current vaccine development, *RevInst Med Trop Sao Paulo*, 2018, **60**, e70, doi: 10.1590/S1678-9946201860070.

12 Lambert M H and Carr S F, The impact of protease inhibitors on the treatment of HIV infection, *Biochem Soc Trans*, 2005, **33**(3), 519-523, doi: 10.1042/BST0330519.

13 Jacobson I M, Gordon S C, Kowdley K V, Yoshida E M, Rodriguez-Torres M, *et al.*, POSITRON study; FUSION study. sofosbuvir for hepatitis C genotype 2 or 3 in patients without treatment options, *N Engl J Med*, 2013, **368**(20), 1867-1877, doi: 10.1056/NEJMoa1214854.

14 Kelley L A, Mezulis S, Yates C M, Wass M N and Sternberg M J, The Phyre2 web portal for protein modeling, prediction and analysis, *Nat Protoc*, 2015, **10**(6), 845-858, doi: 10.1038/nprot.2015.053.

15 Heo L, Park H and Seok C, Galaxy refine: Protein structure refinement driven by side-chain repacking, *Nucleic Acids Res*, 2013, **41**(Web Server issue), W384-W388, doi: 10.1093/nar/gkt458.

16 Hollingsworth S A and Karplus P A, A fresh look at the Ramachandran plot and the occurrence of standard structures in proteins, *Biomol Concepts*, 2010, **1**(3-4), 271-283, doi: 10.1515/BMC.2010.022.

17 Wiederstein M and Sippl M J, ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins, *Nucleic Acids Res*, 2007, **35**(Web Server issue), W407-W410, doi: 10.1093/nar/gkm290.

18 Clifford J N, Høie M H, Deleuran S, Peters B, Nielsen M, *et al.*, BepiPred-3.0: Improved B-cell epitope prediction using protein language models, *Protein Sci*, 2022, **31**(12), e4497, doi: 10.1002/pro.4497.

19 Paul S, Lindestam A C S, Scriba T J, Dillon M B, Oseroff C, *et al.*, Development and validation of a broad scheme for prediction of HLA class II restricted T cell epitopes, *J Immunol Methods*, 2015, **422**, 28-34, doi: 10.1016/ j.jim.2015.03.022.

20 Díaz-Dinamarca D A, Salazar M L, Castillo B N, Manubens A, Vasquez A E, *et al.*, Protein-Based adjuvants for vaccines as immunomodulators of the innate and adaptive immune response: Current knowledge, challenges, and future opportunities, *Pharmaceutics*, 2022, **14**(8), 1671, doi: 10.3390/pharmaceutics14081671.

21 Sabourin M, Tuzon C T, Fisher T S and Zakian VA, A flexible protein linker improves the function of epitope-tagged proteins in *Saccharomyces cerevisiae*, *Yeast*, 2007, **24**(1), 39-45, doi: 10.1002/yea.1431.

22 Saguil A, Kane S F, Lauters R and Mercado M G, Hand-foot-and-mouth disease: Rapid evidence review, *Am Fam Physician*, 2019, **100**(7), 408-414.

23 Esposito S and Principi N, Hand, foot and mouth disease: Current knowledge on clinical manifestations, epidemiology, aetiology and prevention, *Eur J Clin Microbiol Infect Dis*, 2018, **37**(3), 391-398, doi: 10.1007/s10096-018-3206-x.

24 Tan C W, Lai J K F, Sam I C and Chan Y F, Recent developments in antiviral agents against enterovirus 71 infection, *J Biomed Sci*, 2014, **21**, 14, doi: 10.1186/1423-0127-21-14.

25 Falah N, Montserret R, Lelogeais V, Schuffenecker I, Lina B, *et al.*, Blocking human enterovirus 71 replication by targeting viral 2A protease, *J Antimicrob Chemother*, 2012, **67**(12), 2865-2869, doi: 10.1093/jac/dks304.

26 Smart D, Filippi I, Blume C, Smalley B, Davies D, *et al.*, Rhinovirus 2A is the key protease responsible for instigating the early block to gene expression in infected cells, *J Cell Sci*, 2020, **133**(1), jcs232504, doi: 10.1242/jcs.232504.

27 Ullrich S and Nitsche C, The SARS-CoV-2 main protease as drug target, *Bioorg Med Chem Lett*, 2020, **30**(17), 127377, doi: 10.1016/j.bmcl.2020.127377.

28 Enayatkhani M, Hasaniazad M, Faezi S, Gouklani H, Davoodian P, *et al.*, Reverse vaccinology approach to design a novel multi-epitope vaccine candidate against COVID-19: An *in silico* study, *J Biomol Struct Dyn*, 2021, **39(**8), 2857-2872, doi: 10.1080/07391102.2020.1756411.

29 Le T T V and Do P C, Molecular docking study of various *Enterovirus-A71* 3C protease proteins and their potential inhibitors, *Front Microbiol*, 2022, **13**, 987801, doi: 10.3389/fmicb.2022.987801.

30 Kaul R, Paul P, Kumar S, Büsselberg D, Dwivedi V D, *et al.*, Promising antiviral activities of natural flavonoids against SARS-CoV-2 targets: Systematic review, *Int J Mol Sci*, 2021, **22**(20), 11069, doi: 10.3390/ijms222011069.

31 Lalani S and Poh C L, Flavonoids as antiviral agents for *Enterovirus A71* (*EV-A71*), *Viruses*, 2020, **12**(2), 184, doi: 10.3390/v12020184.

32 Xu L, Su W, Jin J, Chen J, Li X, *et al.*, Identification of luteolin as enterovirus 71 and coxsackievirus A16 inhibitors through reporter viruses and cell viability-based screening, *Viruses*, 2014, **6**(7), 2778-2795, doi: 10.3390/v6072778.

33 Chen J, Xu L, Sun S, Zhang H, Ma T, *et al.*, Identification of cinobufagin and resibufogenin as inhibitors of enterovirus 71 infection, *Chem Res Chin Univ*, 2014, **30**, 953–958, doi: 10.1007/s40242-014-4133-8.

34 Wang L, Wang J, Wang L, Ma S and Liu Y, Anti-Enterovirus 71 agents of natural products, *Molecules*, 2015, ***20***, 16320-16333, doi: 10.3390/molecules200916320.

35 Zhang W, Qiao H, Lv Y, Wang J, Chen X, *et al.*, Apigenin inhibits enterovirus-71 infection by disrupting viral RNA association with trans-acting factors, *PLoS One*, 2014, **9**(10), e110429, doi: 10.1371/journal.pone.0110429.

36 Alizadeh M, Amini-Khoei H, Tahmasebian S, Ghatrehsamani M, Ghatreh Samani K, *et al.*, Designing a novel multi-epitope vaccine against Ebola virus using reverse vaccinology approach, *Sci Rep*, 2022, **12**(1), 7757, doi: 10.1038/s41598-022-11851-z.

37 Aziz S, Almajhdi F N, Waqas M, Ullah I, Salim M A, *et al.*, Contriving multi-epitope vaccine ensemble for monkeypox disease using an immunoinformatics approach, *Front Immunol*, 2022, **13**, 1004804, doi: 10.3389/fimmu. 2022.1004804.

38 Mahmoodi S, Amirzakaria J Z and Ghasemian A, *In silico* design and validation of a novel multi-epitope vaccine candidate against structural proteins of Chikungunya virus using comprehensive immunoinformatics analyses, *PLoS One*, 2023, **18**(5), e0285177, doi: 10.1371/journal. pone.0285177.