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Research Article

In silico characterisation, homology modelling and molecular docking of nervous necrosis virus capsid protein

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Abstract

Viral nervous necrosis (VNN), caused by the nervous necrosis virus (NNV), is one of the marine finfish culture's most serious viral diseases. There are no successful and proven treatment measures to control this infection. *In silico* approach aids in identifying novel antiviral compounds that can be analyzed further to fight VNN infection. The present study aimed to build a 3D model of the NNV capsid protein by *in silico* approach and explore the protein-ligand binding of various commercial and herbal antiviral compounds by molecular docking. The protein sequence NNV capsid protein was retrieved from the UniProt database and analyzed its structural and functional properties. The homology modelling was carried out using SWISS-MODEL. The 3D structure of capsid protein was used as a receptor in HEX docking software against antimicrobial herbal and commercial antiviral compounds taken as ligands. The capsid protein was soluble, stable, hydrophobic and thermostable. The predicted model's Z-value was acceptable as it lies within the range of identical native protein scores. In molecular docking, the top antimicrobial herbal compounds that gave the least E-value were myricitrin (*Myrica nagi)* >nimbin (*Azadirachta indica)* > oleuropein (*Olea polygama)* and the top commercial compounds with the least E-value were monensin> bafilomycin A1 > chloroquine. The docking results showed that these herbal compounds can be used as potential anti-NNV agents in marine aquaculture.

Key words: Capsid protein, Fish disease, Homology modelling, Molecular docking, Viral nervous necrosis

Highlights

- Effective drugs are essential to comprehend the nervous necrosis virus (NNV) in marine aquaculture.
- This study portrayed the importance of bioinformatics in viral drug discovery for use in aquaculture.
- Bioinformatic approaches using NNV capsid protein were able to identify potential herbal compounds to mitigate infection.
- The herbal compounds like myricitrin, nimbin and oleuropein had anti-NNV potential.

INTRODUCTION

The aquatic counterpart of agriculture, called aquaculture, has proliferated worldwide, and today, it produces almost as many fish and shellfish (FAO, 2020). The disease issues are of great concern in fish production. Aquaculture production faces substantial economic losses affecting its growth due to infectious diseases and viral diseases play a significant role, causing huge annual losses. Among the fish viral diseases, betanodavirus infection is the most significant concern (Shetty *et al.*, 2012; Doan *et al.*, 2017; Chang and Pakingking, 2022). Betanodavirus named nervous necrosis virus (NNV) causes a serious infectious disease called Viral nervous necrosis (VNN), which is recognized as a crucial problem worldwide in marine aquaculture (Bandín and Souto, 2020). The VNN, also known as viral encephalopathy and retinopathy (VER), has been observed in at least 120 fish species globally. Economically important species, such as European sea bass (*Dicentrarchus labrax*), Asian sea bass (*Lates calcarifer)*, groupers (*Epinephelus* spp.),

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striped jack *(Pseudocaranx dentex*), Senegalese sole (*Solea senegalensis*), turbot (*Scophthalmus maximus*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) are susceptible to VNN (Doan *et al.*, 2017; Toffan *et al.*, 2017; Chang and Pakingking, 2022). It mainly affects the fish's central nervous system and retina, causing characteristic necrotic lesions and vacuolation (Liu *et al.*, 2016). It comes under the family Nodaviridae and the genus Betanodavirus (Jia *et al.*, 2015). It is a non-enveloped RNA virus with icosahedral symmetry of 20-30 nm diameter and has two positive-sense RNA strands named RNA1 and RNA2. RNA1 encodes a mitochondrial enzyme, the rRNA-dependent RNA polymerase (RdRp), accountable for viral replication (Jia *et al.*, 2015). The role of apoptosis is performed by the NNV capsid protein encoded by RNA2 (Bandín and Souto, 2020). RNA3, RNA1 subgenome encodes for two non-structural proteins, B1 and B2. Protein B1 is expressed at the early stage of infection, whereas the most conserved protein among the betanodaviruses is protein B2. Recently, an RNAi antagonist was determined (Su *et al.*, 2014).

From a phylogenetic analysis of capsid protein, betanodaviruses were grouped into four different species, namely the tiger puffer nervous necrosis virus (TPNNV) type, the striped jack nervous necrosis virus (SJNNV) type, barfin flounder nervous necrosis virus (BFNNV) type and the red-spotted grouper nervous necrosis virus (RGNNV) type (Panzarin *et al.*, 2016). Capsid proteins that safeguard the viral genomes during entry and exit from the host cells can modulate the specificity and activity of viral replication complexes. It is the structural protein generally referred to as the head of the virus, protecting its nucleic acid in a closed shell. It is a structural protein of betanodavirus that is proven to be an attachment receptor and a vital target for treating VNN infection (Huang *et al.*, 2020). New effective treatment measures must be identified soon to reduce the economic losses caused by betanodavirus in marine aquaculture. The present work aimed at exploring the physiochemical, functional and secondary structure properties of the NNV capsid protein, homology modelling of protein, and identifying the best antiviral compound out of herbal and commercial antivirals through *in silico* approach and molecular docking.

MATERIALS AND METHODS

Sequence retrieval: The FASTA format of the NNV capsid protein sequence (accession number: D5LG60) was recovered from the UniPort protein database (https://www.uniprot.org/) to characterise its properties and for further study.

Prediction of physicochemical properties: ExPASy-Prot Param prediction server [https://web.expasy.org/protparam/] was used to calculate the physicochemical properties of NNV capsid protein, such as amino acid composition, number of amino acids, molecular weight, extinction coefficient [EC] (Gill and von Hippel, 1989), aliphatic index [AI] (Ikai, 1980), instability index [II] (Guruprasad *et al.*, 1990), grand average hydropathy [GRAVY] (Kyte and Doolittle, 1982), isoelectric point (pI), molecular formula, the total number of atoms, number of positively and negatively charged residues.

Functional analysis: The nature of the protein, i.e., soluble or transmembrane was identified by the SOSUI server [http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_ submit. html (Hirokawa *et al.*, 1998). The presence of disulphide bonds, bonding patterns, and cysteine residues was predicted with the help of the CYS_REC tool [http://linux1.softberry.com].

Secondary structure characterization: The secondary structure is an intermediate before the protein folds into its three-dimensional tertiary structure. SOPMA (self-optimized prediction method with alignment) tool was used to acquire the secondary structures like

alpha-helix, extended strand, beta-turn and the random coil of NNV capsid protein (Combet *et al.*, 2000) [https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html].

Homology modelling: Using template structure from PDB, the protein structural database, a fully automated protein structure homology-modelling server called the SWISS-MODEL server carried out the homology modelling of the 3D structure of NNV capsid protein. It was accessed using the ExPASy web server [https://swissmodel.expasy.org/], which identified the structural template and aligned the target sequence with the template structure. With the help of a template, it built the model and analyzed the quality.

Evaluation of tertiary structure: The qualities of the predicted models were analyzed by RAMPAGE (Lovell *et al.*, 2003), ProQ (Cristobal *et al.*, 2001), ProSA-web (Sippl, 1993) and Ramachandran plot (Ramachandran *et al.*, 1963).

Molecular docking: The NNV capsid protein was an essential component of infectious virions. Since capsid proteins play a vital role in the entry and exit of viruses, induction of cell death in the host and their structural property, they are considered a drug target. The antimicrobial herbal compounds from various sources and commercially important antiviral compounds were chosen as ligands to dock against the NNV capsid protein. The chemical database retrieved their three-dimensional chemical structures from PubChem [https://pubchem.ncbi.nlm.nih.gov/]. The list of retrieved compounds and their Pubchem ID is tabulated in Table 1. The 3D structures of the selected herbal compounds having antimicrobial properties were retrieved from PubChem, a public repository database of chemical molecules and their biological activities (Kim *et al.*, 2016). The 3D chemical structures were downloaded in Structure data file (SDF) format. The software Open Babel was used for converting the ligand from SDF format to PDB format as the molecular docking requires the target (receptor) and ligand in the form of a PDB file. HDOCK (Yan *et al.*, 2020) and HEX 8.0.0.0 Cuda (Macindoe *et al.*, 2010) were used for docking the PDB structure of the receptor and ligands.

RESULTS

Physicochemical properties prediction: As summarised in Table 2, the NNV capsid protein had 338 amino acids. The computed pI value

Table 1. The herbal and commercial antiviral compounds retrieved from PubChem for molecular docking

Antimicrobial herbal compounds	Pubchem ID	Commercial antiviral compounds	Pubchem ID
Quercetin	5280343	Idoxuridine	5905
Anthraquinone	6780	LJ001	49777349
Madecassic acid	73412	Ribavirin	37542
Zingerone	31211	Chlorpromazine hydrochloride	6240
Nimbin	108058	Chloroquine	2719
Curcumin	969516	bafilomycin A1	6436223
Gymnemagenol	21592406	Monensin	441145
Dasyscyphin C	11562458	Furan-2-yl acetate	12719907
Myricitrin	5281673	Amantadine	2130
Oleuropein	5281544	Tenofovir	464205

Table 2. Physiochemical properties of nervous necrosis virus capsid protein

Number of amino acids	338
Molecular weight	37030.79
Theoretical pI	9.01
Total number of negatively charged residues (Asp + Glu)	33
Total number of positively charged residues (Arg + Lys)	38
Molecular formula	$C_{1637}H_{2575}N_{471}O_{495}S_8$
Total number of atoms	5186
Extinction coefficients at 280 nm/M/cm	57660
Instability index (II)	38.88
Aliphatic index	78.46
Grand average of hydropathicity (GRAVY)	-0.343

of capsid protein was 9.01, indicating that it is alkaline. The capsid protein had a total positive charge residue (Arg+Lys) of 33 and negative charge residues (Asp+Glu) of 38. The NNV capsid protein extinction coefficient measured at 280 nm was 57660M^{-1} cm^{-1}. The stability of the protein was indicated by the instability index of 38.88. The AI value of 78.46 stated the thermostability of the capsid protein. The GRAVY value of -0.343 affirmed the hydrophobic and non-polar nature of the NNV capsid protein. The NNV capsid protein amino acid composition, as computed using ExPASyProtParam, is tabulated in Table 3. It contained a higher proportion of threonine (9.2% and leucine (8.6%).

Functional analysis: The NNV capsid protein was soluble. The transmembrane region of the protein could not be detected. The capsid protein had four cysteines, as revealed by the CYS_REC tool, and none formed a disulphide bond, a measure of protein stability, indicating the protein is not stronger to a great extent.

Secondary structure characterization: The alpha helix constituted 13.91% of NNV capsid protein. The extended strand composition of the capsid protein was 26.04%. Most amino acids contribute to the random coils. About 52.37% of random coils were found in NNV capsid protein. On the other hand, beta turns constituted 7.69% (Table 4). The capsid protein lacked other secondary structures like 3_{10} helix, pi helices, Beta Bridge, bend region, ambiguous states and other states.

Table 3. Amino acid composition of nervous necrosis virus capsid protein

Amino acid	Composition (%)	Amino acid	Composition (%)
Alanine	8.3	Leucine	8.6
Arginine	7.7	Lysine	3.6
Asparagine	4.7	Methionine	1.2
Aspartic acid	6.5	Phenylalanine	3.3
Cysteine	1.2	Proline	6.5
Glutamine	3.3	Serine	6.8
Glutamic acid	3.3	Threonine	9.2
Glycine	8.3	Tryptophan	2.4
Histidine	1.2	Tyrosine	2.7
Isoleucine	3.3	Valine	8.3

Table 4. Secondary structure elements of nervous necrosis virus capsid protein computed using the SOPMA tool

Composition	Number of amino acids	Secondary structure (%)
Alpha-helix	47	13.91
Extended strand	88	26.04
Beta-turn	26	7.69
Random coil	177	52.37

Homology modelling: The 3D model was built for the NNV capsid protein using the Swiss Model server based on the similarity between the identified template and the sequence. The model obtained with the best sequence identity is given in Fig. 1a. The resultant data of the capsid protein by SWISS-MODEL accessed via the ExPASy web server are tabulated in Table 5. The capsid protein was built with the template 4wiz.3.A described as coat protein at a resolution of 3.60Å. The Global Model Quality Estimation score (GMQE) of the NNVcapsid protein was 0.94, which indicated that the model is of the best quality. The QMEAN Z-score of NNVcapsid protein was -2.45.

Table 5. Homology modelling data of nervous necrosis virus capsid protein by SWISS-MODEL accessed via the ExPASy web server

Characteristics	Capsid protein
Template	4wiz.3.A
Description	Coat protein
Resolution	3.60Å
GMQE	0.94
QMEAN	-2.45

Evaluation of tertiary structure: The accuracy of the predicted model was further verified using RAMPAGE analysis (OR), Protein Quality Predictor (ProQ), ProSA-web-Protein Structure Analysis and Ramachandran plot. The RAMPAGE - Ramachandran plot analysis results indicated that the favoured region of NNV capsid protein has 97.4% residues. In contrast, the allowed region has 2.6% residues, thus, adding up to the better quality of the model. The outlier region of NNV capsid protein had no residues. The ProQ validation results of the predicted model revealed by the capsid protein's LG score (4.478) and Maxsub value (0.289) indicated a reasonably good quality model. The results of the ProSA-web validation on the predicted model are represented in Figs. 1b and 1c. Output Z-score indicated the overall quality of the model. In the ProSA web server, the predicted model of NNV capsid protein showed a Z-score of -7.44 (Fig. 1c). Ramachandran plot of NNV capsid protein was determined with the help of SWISS-MODEL via the ExPASy web server and depicted in Fig. 1d.

Molecular docking: The NNV capsid protein, as a receptor, was docked with antimicrobial herbal and commercial antiviral compounds, which were used as ligands using HEX 8.0.0.0 Cuda software. The docking results are tabulated in Table 6. The compounds with the lowest net binding free energy have the highest inhibition. The molecular docking and interactions between the capsid protein and ligands such as myricitrin, nimbin, monensin and bafilomycin A1 are represented pictorially using the HDOCK server in Fig. 2 (a,c,e,g). The amino acid labelling of interactions between the respective ligands on the capsid protein of NNV as predicted by the HDOCK server are depicted in Fig. 2 (b,d,f,h).

DISCUSSION

As per the Prot Param tool results, the NNV capsid protein had 338 amino acids. The pI value was in the alkaline range. Łapińska *et al.* (2017) opined that the pI value would be helpful for the purification of protein using isoelectric

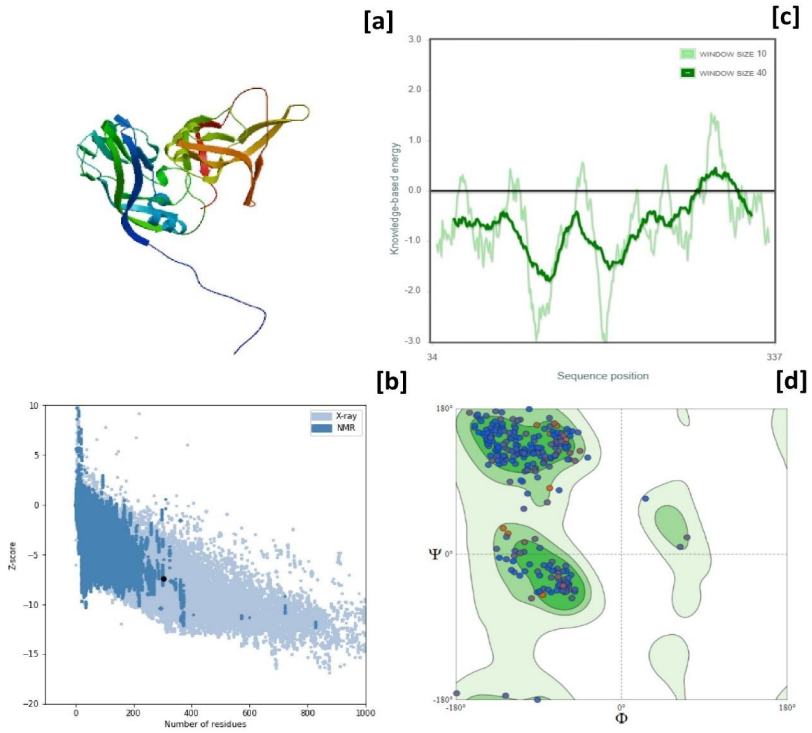

Fig. 1. The nervous necrosis virus capsid protein (a) homology model by SWISS-MODEL, (b) ProSA web Z-score, (c) plot of Z- score and (d) Ramachandran plot

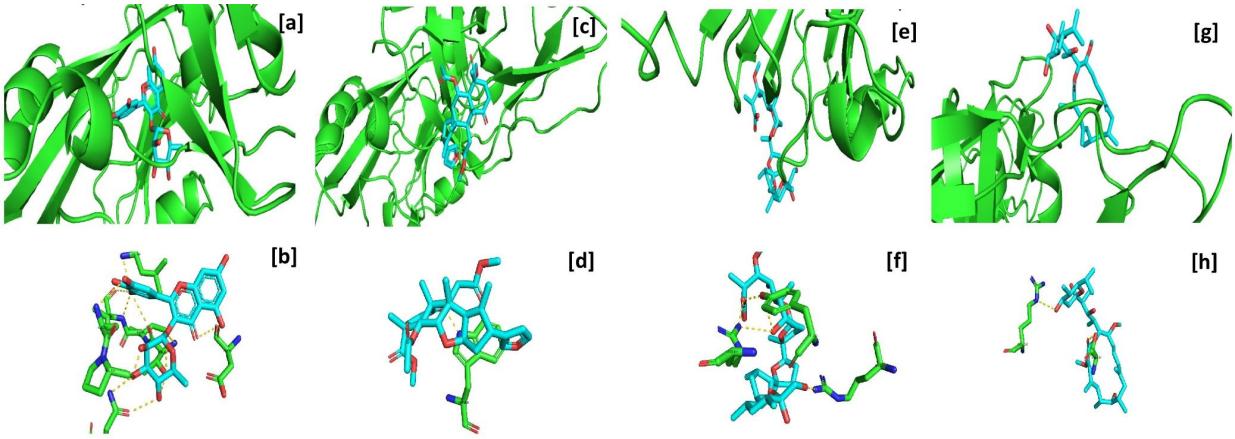

Fig. 2. Molecular docking and interaction of nervous necrosis virus capsid protein with antimicrobial herbal compounds [a] myricitrin and [b] interacting amino acid residues - Asp81, Thr83, Ile 84, Val85, Leu88, Gln175, Ala220, Pro221, and [c] nimbin and [d] interacting amino acid residue- Trp280, and commercial antiviral compounds [e] monensin and [f] interacting

focusing on a 2D gel. The extinction coefficient was measured at 280 nm because of the maximum absorbance of protein due to the presence of aromatic amino acids such as tyrosine and tryptophan, and a minor portion of phenylalanine (Lakowicz, 2013). The computed extinction coefficient values are used to quantitatively study the protein-ligand and

Table 6. Molecular docking of nervous necrosis virus capsid protein against various herbal and commercial antiviral compounds using the HEX tool

Antimicrobial herbal compounds	E-value (ΔG_{bind})*	Commercial antiviral compounds	E-value (ΔG_{bind})*
Myricitrin	-304.10	Monensin	-304.56
Nimbin	-280.66	Bafilomycin A1	-290.54
Oleuropein	-264.54	Chloroquine	-248.39
Dasyscyphin C	-255.83	Chlorpromazine hydrochloride	-233.19
Curcumin	-254.19	LJ001	-231.41
Madecassic acid	-253.02	Ribavirin	-216.18
Quercetin	-241.95	Idoxuridine	-213.53
Gymnemagenol	-237.59	Tenofovir	-197.32
Zingerone	-208.79	Furan-2-yl acetate	-160.16
Anthraquinone	-203.86	Amantadine	-125.29

*ΔG_{bind}: Net binding free energy

protein-protein interaction in solution. The stability of the protein in a test tube is determined by the instability index (Ghosh *et al.*, 2017). A protein with an instability index of less than 40 is expected as stable (Guruprasad *et al.*, 1990; Enany, 2014). The aliphatic index (AI) measures the thermostability of proteins. It is based on the relative volume occupied by aliphatic side chains of amino acids. The thermostability of NNV capsid protein (78.46) was suitable, as the higher the aliphatic index, the better the thermostability (Ikai, 1980; Juibari *et al.*, 2019). The hydrophobicity value of a peptide was represented by the grand average of hydropathy (GRAVY) value, which provided the interaction of a particular protein with water. The lower GRAVY index values indicated better interaction.

The SOSUI server (Hirokawa *et al.*, 1998) was used in the identification of the type of protein and to identify the presence or absence of transmembrane regions of the proteins. The soluble proteins are present in the cytoplasm, mitochondria and nucleus. On the other hand, membrane proteins are found anchored or buried on the membrane. The presence of disulphide bonds in the protein measures its stability. The number of residues between the linked cysteines is directly proportional to the particular disulphide bond, enhancing the stability of the protein. The absence of disulphide bonds in cysteines, as per the CYS_REC tool, suggested that these proteins are not stronger to a great extent. The secondary structure composition was analyzed by the Self-Optimized Prediction Method with Alignment (SOPMA) server (Combet *et al.*, 2000). Among all components, random coils and extended strands were predominant in NNV capsid protein, followed by the alpha helices and beta-turn.

The SWISS-MODEL server was used for the homology modelling of NNV capsid protein using the template structure from the protein structural database (PDB). The score between 0 and 1 which unites the properties of the target-template alignment and the template search method that depicts the quality of the protein model is called GMQE score (Waterhouse *et al.*, 2018). Generally, the higher the numbers, the maximum the reliability. The QMEAN is a composite estimator, which gives both global (i.e., for the entire structure) and local (i.e., per residue) absolute quality estimates as one single model based on the different geometrical properties (Benkert *et al.*, 2008). The model was predicted to be good as per the QMEAN Z-score, which compared the interaction potential between all atoms, Cβ atoms only, torsion angle

and solvation potential. According to Shaik *et al.* (2019), the model and experimental structures of similar size are in good agreement when the QMEAN Z-score is around zero.

Though the SWISS-MODEL had some inbuilt quality estimate scores, there are plenty of online tools to check the quality of the protein model built. The dihedral angles formed due to alpha-helix and beta-sheet conformations are included in the favoured regions in RAMPAGE analysis. The ~98.0% of residues in the favoured region and ~2.0% in the allowed region are expected for a good protein tertiary structure (Basu, 2022). The allowed regions correlate with conformations where there are no steric clashes. The steric clashes rise when there is an unnatural overlap of any two non-bonding atoms in a protein structure (Ramachandran *et al.*, 2011). The results indicated the better quality of the NNV capsid protein. The RAMPAGE evaluated the residues percentage in the favoured and allowed regions and the percentage in the outlier region. The outlier residues are the ones that do not have stable phi (φ) and psi (Ψ) angle values and, thus, they are predicted to be unstable in their conformation. The ProQ validated a protein based on several structural features and predicted the quality of a protein model. The LG score and MaxSub values of ProQ indicated the good quality of NNV capsid protein. ProSA-web, an easy-to-use interface server (Sippl, 1993; Wiederstein and Sippl, 2007), was employed in the structure validation of NNV capsid protein. The X-ray crystallography (light blue) and Nuclear Magnetic Resonance spectroscopy (NMR) (dark blue) determined the Z-score of the ProSA web by their length and specified in the form of an image (Pathak *et al.*, 2014). A positive value of the Z-score may indicate an error in protein structure. The other two ways of representation depend on the Z-score predicted. It depicted the exact position of the Z-score in the image, predominantly of negative values, thus indicating the good quality of the anticipated models. The ProSA-web server also gave the plot of residue scores. It depicted the arrangement of amino acid residues, which also resulted in the average overall quality of the models. The plot of the torsional angles of the amino acids is called the Ramachandran plot. Torsional angles include phi (φ) and psi (Ψ) angles of residues in a peptide (Hollingsworth and Karplus, 2010). The Ramachandran plot analysis indicated that the quality of the experimental structures was much higher, as revealed by the residues in the favoured regions (green), allowed regions (light green), generously allowed regions (light blue) and disallowed regions (white).

Molecular docking is the study of the interaction between two or more molecular structures (e.g., protein, enzyme and drug) and how they fit with each other. It has emerged as an essential tool for drug discovery (Azam and Abbasi, 2013). HDOCK and HEX 8.0.0.0 Cuda software were used for docking. HDOCK server provides the amino acid labelling of target ligand interaction whereas HEX 8.0.0.0 Cuda software combines the ligand and receptor with optimized conformation and gives results in less binding free energy. The lesser the net binding free energy, the stronger the docking and considered the best ligand for the target protein. In this study, the top antimicrobial herbal compounds that gave the least binding free energy (ΔG_{bind}) or E-value were myricitrin (-304.10) from bayberry, *Myrica nagi*, followed by nimbin (-280.66) from neem, *Azadirachta indica*, oleuropein (-264.54) from olive, *Olea polygama* and dasyscyphin C (-255.83) from false daisy *Eclipta prostrate*. Viral haemorrhagic septicaemia virus infection was successfully controlled by oleuropein (Ole), a significant compound in olive tree leaf (*Olea europaea*) extract (Micol *et al.*, 2005). The antiviral activity of dasyscyphin C extract of leaves of *Eclipta prostrata* was demonstrated against grouper nervous necrosis virus (GNNV) *in vitro* (Krishnan *et al.*, 2010). Gymnemagenol from *Gymnema sylvestre* leaf extract inhibited the proliferation of GNNV to 53% under *in vitro* conditions in SIGE cell lines (Khanna *et al.*, 2011). Curcumin exhibited potent antiviral

potential against Herpes simplex viruses 1 and 2 (Zandi *et al.*, 2010). Myricetin has shown antibacterial and antiviral properties against diverse infectious agents. It is a potent inhibitor of reverse transcriptase from human immunodeficiency virus (HIV) and Rauscher murine leukaemia virus (RLV). An aqueous neem plant bark extract acted as a strong entry inhibitor against HSV 1 infection into natural target cells (Tiwari *et al.*, 2010).

The docking scores of commercial compounds seemed to be more or less similar to herbal compounds. The top commercial antiviral compounds that gave the least binding free energy (ΔG_{bind}) or E-value were monensin (-304.56), followed by bafilomycin A1 (-290.54), chloroquine (-248.39) and chlorpromazine hydrochloride (-233.19). The lysosomotropic agents such as monensin and bafilomycin A1 inhibited betanodavirus. In an earlier study, the entry of betanodaviruses into cells was inhibited by the presence of 1 mM NH_4Cl or 1µM chloroquine in the medium (Adachi *et al.*, 2007). LJ001 is a lipophilic thiazolidine derivative that inhibits numerous enveloped viruses' entry by specific inhibition of virus-cell fusion (Vigant *et al.*, 2013). The antiviral activity of ribavirin against RSV infection, hepatitis C and some viral hemorrhagic fevers have been reported (Ramírez-Olivencia *et al.*, 2019). Tenofovir is used to treat acquired immunodeficiency syndrome (AIDS) caused by HIV (Fernandez-Fernandez *et al.*, 2011). The compounds like monensin, bafilomycin A1 and chloroquine exhibited the lowest net binding free energy against NNV, which can be explored for its mitigation in commercial aquaculture. Antimicrobial herbal compounds such as myricitrin, nimbin, oleuropein and curcumin have shown better docking scores similar to commercial antiviral compounds.

Nowadays, bioinformatics plays a crucial role in novel drug discovery by reducing time and expenses by utilising innovations in technology to analyse the data virtually through a computational approach. This study, thus, portrayed the importance of bioinformatics in viral drug discovery for use in aquaculture. The *in-silico* characterization and 3-D structure prediction of NNV capsid protein led us to know about the keen properties of the protein which are the basics for drug discovery and its interaction. Moreover, the comparative study on molecular docking with commercial antiviral and antimicrobial herbal compounds showed its ability to interact with the virulent protein of NNV. This study gave the baseline data on the potential use of herbal compounds with antimicrobial properties to overcome the use of synthetic commercial antiviral drugs to mitigate VNN infection. Hence, these herbal compounds can be explored on large scale by *in-vitro* and *in-vivo* assays at field level to combat VNN infection in marine finfish aquaculture.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: TC, SK: Data collection and analysis; BNR: Interpretation of data and preparation of the draft manuscript; TJA: Supervision of research and finalization of the manuscript.

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