Babol University of Medical Sciences
IJMCM
International Journal of Molecular and Cellular Medicine
p-ISSN: 2251-9637 o-ISSN: 2251-9645
Cellular and Molecular Biology Research Center

In Silico and In vitro Evaluations of the Antibacterial Activities of HIV-1 Nef Peptides against *Pseudomonas aeruginosa*

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Article type:
Original Article

Received:
2022.11.15
Revised:
2024.05.21
Accepted:
2024.05.29

ABSTRACT

One of the burning issues facing healthcare organizations is multidrug-resistant (MDR) bacteria. *P. aeruginosa* is an MDR opportunistic bacterium responsible for nosocomial and fatal infections in immunosuppressed individuals. According to previous studies, efflux pump activity and biofilm formation are the most common resistance mechanisms in *P. aeruginosa*. The aim of this study was to propose new antimicrobial peptides (AMPs) that target *P. aeruginosa* and can effectively address these resistance mechanisms through *in silico* and *in vitro* assessments. Since AMPs are an attractive alternative to antibiotics, in vitro experiments were carried out along with bioinformatics analyses on 19 Nef peptides (derived from the HIV-1 Nef protein) in the current study. Several servers, including Dbaasps, Antibp2, CLASSAMP2, ToxinPred, dPABBs and ProtParam were used to predict Nef peptides as AMPs. To evaluate the binding affinities, a molecular docking analysis was performed with the HADDOCK web server for all Nef peptide models against two effective proteins of *P. aeruginosa* (MexB and PqsR) that play a role in efflux and quorum sensing. Moreover, the antibacterial and antibiofilm activity of the Nef peptides was investigated in a resistant strain of *P. aeruginosa*. The results of molecular docking revealed that all Nef peptides have a significant binding affinity to the abovementioned proteins. Nef-Peptide-19 has the highest affinity to the active sites of MexB and PqsR with the HADDOCK scores of -136.1 ± 1.7 and -129.4 ± 2, respectively. According to the results of *in vitro* evaluation, Nef peptide 19 showed remarked activity against *P. aeruginosa* with minimum inhibitory and bactericidal concentrations (MIC and MBC) of 10 µM and 20 µM, respectively. In addition, biofilm inhibitory activity was observed at a concentration of 20 µM. Finally, Nef peptide 19 is proposed as a new AMP against *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, antibacterial agents, antimicrobial peptides, molecular docking simulation, biofilms, bacterial infections

Cite this article: Koosehlar E, et al. *In Silico* and *In vitro* Evaluations of the Antibacterial Activities of HIV-1 Nef Peptides against *Pseudomonas Aeruginosa* . *International Journal of Molecular and Cellular Medicine*. 2024; 13(1):46-63. **DOI:** 10.22088/IJMCM.BUMS.13.1.46

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[DOI: 10.22088/IJMCM.BUMS.13.1.46]

Introduction

Nowadays, due to the overuse and misuse of antibiotics, a fundamental challenge for researchers is to discover new therapeutics to counteract emerging bacterial resistance (1). A systematic study (in 2022) estimated that in 2019, 1.27 million deaths occurred in 204 countries due to bacterial resistance (2). According to reports from the World Health Organization (WHO), *P. aeruginosa* is one of the greatest threats to human health (3). The mortality rate in patients with *P. aeruginosa* infections is 18 to 61 percent (4). This bacterium causes an opportunistic nosocomial infection that occurs in cystic fibrosis patients due to the formation of a biofilm in their lungs (5). *P. aeruginosa* is resistant to various antibiotics such as ceftazidime, gentamicin and ciprofloxacin (6). The low permeability of its cell wall, which is 100 times more impermeable than that of *Escherichia coli* (7), the high expression of efflux pumps and biofilm formation are some of the reasons for the resistance of this bacterium (8).

The efflux pump system (EPS) such as mexCD-oprJ, mexXY-oprM, mexEF-oprN and mexAB-oprM play a crucial role in the resistance of *P. aeruginosa* by expelling toxic substances, including antibiotics, from the cell (9) (10). Overexpression of mexAB-oprM leads to a marked development of MDR strains of *P. aeruginosa* (11). EPS consists of three components: the proton motive force (MexB), outer membrane agent (OprM) and periplasmic adhesion protein (MexA) (12). The EPS can be inhibited by interfering with the regulatory mechanism against its overproduction, blocking the pump outlets (OprM), altering the structure of antibiotics and disrupting the assembly of efflux pump autoinducers by EP inhibitors (EPIs). A molecular docking study indicates that some antibiotics act as competitive inhibitors of MexB and determine the active amino acids (10).

Quorum sensing (QS) and biofilm formation are other mechanisms responsible for the adaptability and resistance of *P. aeruginosa* (13). This organism is a typical example of biofilm production (14). QS plays a role in biofilm production and the use of QS inhibitors (QSIs) could be an excellent way to control MDR bacteria (15). The QS systems of *P. aeruginosa* consist of the RhlI/RhIR, LasI/LasR and Pseudomonas quinolone signal (PQS) types (16). PqsR, also known as MvfR (Multiple virulence factor Receptor), is an essential multifunctional receptor in the PQS. Suppression of PqsR disrupts QS signaling and subsequently inhibits the production of virulence factors and biofilm (17). Antimicrobial peptides (AMPs) have been recognized as biofilm suppressors and can reduce the development of resistance (18).

AMPs differ in many ways but have some common features (19). For example, AMPs typically consist of less than 100 amino acids, mainly have an α-helical configuration and are mostly cationic (20). AMPs are inherently amphipathic and hydrophobic, which enables them to interact more strongly with the bacterial membrane (21). Scientists have found AMPs in various organisms (mammals, amphibians, aquatic animals, insects and plants) and microorganisms (protozoa, fungi and bacteria) (22).

More importantly, the researchers have discovered new AMPs in protein sequences of some viruses. For instance, the peptides HBc ARD and pepR are isolated from hepatitis B virus (HBV) and the capsid of Dango virus, respectively (23, 24). Therefore, the current study utilized a bioinformatics approach to investigate 19 peptides from the HIV-1 Nef protein (Nef peptides) for the prediction of antibacterial and antibiofilm activity, estimation of toxicity and half-life, and analysis of molecular interactions. The present study also investigated the inhibitory effect of Nef peptides against *P. aeruginosa* (PTCC 1027) *in vitro*.

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Materials and methods

The materials and methods of the present study comprised two parts: computational and experimental investigations. The workflow, the different methods used in the current study and the results are summarized in a schematic illustration as a draft of the work (Figure 1).

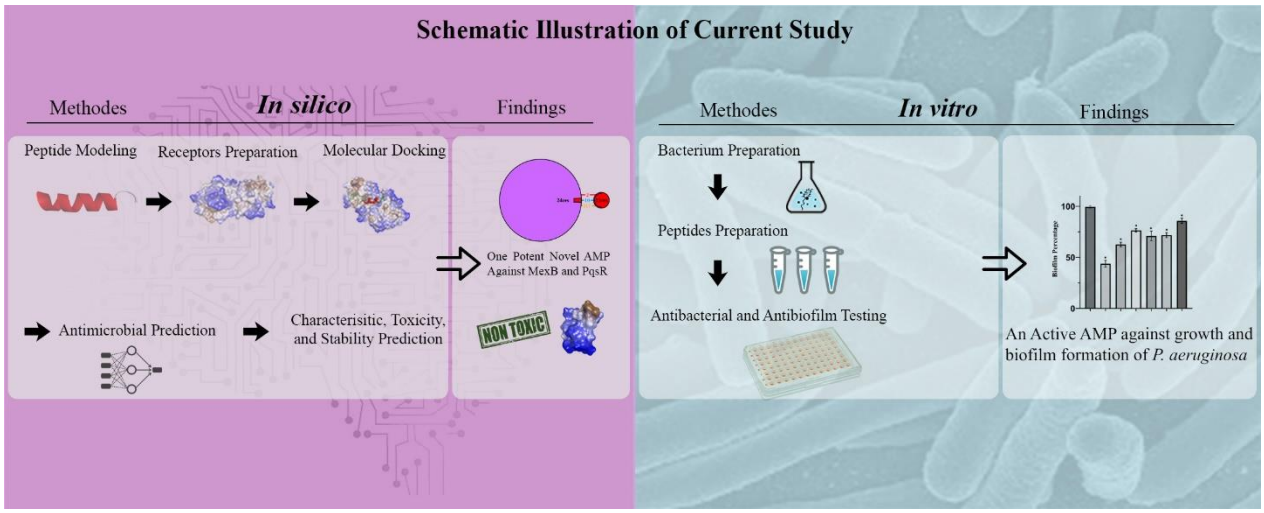

Fig. 1. Illustration of the study workflow.

In silico Studies

3D Structure of Receptors and Nef-Peptides

The 3D structures of the MexB and PqsR proteins were determined from the RCSB database (www.rcsb.org) using PDB ID 6IIA and 4JVD with a resolution of 2.91 and 2.95 Angstroms, respectively. The structures of the Nef peptides were generated from their sequences using *de novo* modeling in the PEP-FOLD$_{3.5}$ server with default parameters (25). The sequences of the Nef peptides are listed in Table 1.

Optimization and Verification of Peptide Models

The best 3D models of the Nef peptides were optimized in 100 steps using the steepest descent algorithm of the Chimera software (version 1.15) (26). Finally, the PDB files of the optimized peptides were uploaded to PROCHECK and ProSA to check Ramachandran plots and Z-score numbers, respectively (27, 28).

Antimicrobial and Antibiofilm Prediction of Nef-Peptides

The Nef peptide sequences were screened for antimicrobial activity using three prediction web servers, including CLASSAMP2 (29), Antibp2 (30), and Dbaasps (31). The dPABBs web server was also used to predict antibiofilm activity (32). Each peptide was scored based on the probability of predicted antimicrobial properties. These servers use a variety of training datasets for machine-learning approaches. Various algorithms were used, including Random Forest (RF), Discriminate Analysis (DA), Support Vector Machine (SVM) and Artificial Neural Network (ANN) (29, 33).

Physicochemical Properties prediction

ProtParam was used to predict critical indices such as GRAVY (34), amphiphilicity, net charge, net hydrogen, and molecular weight. Based on the amino acid composition of each peptide sequence, the

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dPABBs web server predicts the physicochemical properties of each query (32). Furthermore, the percentage of secondary structure in the Nef peptides was analyzed using YASARA software (version 21.12.19) (35).

Table 1. The sequences of all 19 Nef peptides.

Nef-Peptides	Sequences
Nef-Peptide-1	GGKWSKSSVVGWPTVRERMR
Nef-Peptide-2	GWPTVRERMRRAEPAADGVG
Nef-Peptide-3	RAEPAADGVGAASRDLEKHG
Nef-Peptide-4	AASRDLEKHGAITSSNTAAT
Nef-Peptide-5	AITSSNTAATNAACAWLEAQ
Nef-Peptide-6	NAACAWLEAQEEEEVGFPV T
Nef-Peptide-7	EEEEVGFPVTPQVPLRPMTY
Nef-Peptide-8	KAAVDLSHFLKEKGGLEGLI
Nef-Peptide-9	KEKGGLEGLIHSQRRQDILD
Nef-Peptide-10	HSQRRQDILDLWIYHTQGYF
Nef-Peptide-11	LWIYHTQGYFPDWQNYTPGP
Nef-Peptide-12	PDWQNYTPGPGVRYPLTFGW
Nef-Peptide-13	GVRYPLTFGWCYKLVPVEPD
Nef-Peptide-14	CYKLVPVEPDKVEEANKGEN
Nef-Peptide-15	KVEEANKGENTSLLHPVSLH
Nef-Peptide-16	TSLLHPVSLHGMDDPEREVL
Nef-Peptide-17	GMDDPEREVLEWRFDSRLAF
Nef-Peptide-18	EWRFDSRLAFHHVARELHPE
Nef-Peptide-19	HHVARELHPEYFKNC

Toxicity and Stability Estimation

The peptide sequences were checked using the online tools ToxinPred and ProtParam to predict the instability index, half-life and toxicity in mammalian cells (30, 34).

Molecular Docking Analysis

Molecular docking was performed using the HADDOCK web server (HADDOCK2.4) (36, 37), and 19 Nef peptides were docked to MexB and PqsR proteins. The peptides were considered as ligands (labeled in chain B), and MexB and PqsR proteins were selected as receptors (labeled in chain A). Docking was performed with optimized parameters for the peptide-protein complex. For the sampling parameters, the number of structures for rigid body docking was set to 1000, the number of structures for semi-flexible refinement was set to 200, the cutoff parameter for RMSD was set to 5 and water was selected as the explicit solvent.

Peptide-Protein Complex Interaction Analysis

The Discovery Studio Visualizer software and the PDBsum tool were used to analyze the peptide-protein complexes and display the binding residues (38, 39).

***In vitro* Studies**

Bacterium Strain and Nef-Peptide Preparation

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P. aeruginosa (PTCC 1074), also known as ATCC 9027, was obtained from Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. This species was cultured in nutrient broth and agar (NB and NA) for 24 hours at 37 °C, stored at four °C and recultured for subsequent studies. The nineteen Nef peptides were purchased from the Cenetr for AIDS Reagents, National Institute for Biological Standards and Control (NIBSC), UK (with repository reference ARP7074). The solution of Nef peptides was prepared in deionized water at a concentration of 2560 µM based on their molecular weight.

Antibacterial Susceptibility Testing

A 0.5 McFarland turbidity standard (1.5×10^8) was prepared from overnight cultured *P. aeruginosa* PTCC 1074, and added to 96-well microplates (SPL Life Sciences, South Korea). In addition, antibacterial susceptibility was performed and evaluated using the minimum inhibitory concentration (MIC) method. In brief, 100 µl of Nef peptides, 100 µl of NB medium and 50 µl of bacterial suspension were added to each well. The bacterial suspension and NB medium were positive and negative controls, respectively. The lowest concentration at which no growth was seen after overnight incubation at 37 °C was considered the MIC.

Furthermore, the MBC method was used to test bactericidal activity. Ten µl of the wells in which the MIC was observed and 2-fold of the MIC were transferred to a NA and incubated at 37 °C for 24 hours. The MBC is the lowest concentration at which no bacterial colonies form on the NA (40). All tests were done in triplicate.

Antibiofilm Inhibition Evaluation

Biofilm formation was determined using the microtiter plate method to investigate the efficacy of the Nef peptides (41, 42). The *P. aeruginosa* PTCC 1074 suspension was incubated overnight for 48 hours at 37 °C with 20 µM of the best Nef peptides (Nef peptide-1, Nef peptide-10, Nef peptide-14, Nef peptide-16, Nef peptide-18 and Nef peptide-19) in 48-well microplates (SPL Life Sciences, South Korea). After incubation, the microplates were washed with physiological saline to remove the unbound and dead cells and allowed to dry at room temperature for 10 minutes. Then, they were stained by adding 250 µL of 0.1% crystal violet solution for 10 minutes and stored at room temperature to dry and stabilize the biofilm. Finally, 200 µL of 95% ethanol was added for 10 minutes and the qualification of biofilm production was measured using a microplate reader (Awareness Technology ChroMate® Microplate Reader, America) at OD_{595nm}.

Statistical Analysis

The data of treated groups and control were compared by two-way ANOVA with a *p*-value of less than 0.05. These statistical analyses were performed using GraphPad Prism software (version 9.3.1.471, San Diego, CA, USA). All tests were carried out in triplicate, and results were presented as mean ± SD.

Results

Computational Analysis

Nef-Peptide 3D Structure Prediction and Verification

Prediction of the 3D structures of Nef peptides by PEPFOLD3.5 yielded ten models for each Nef peptide. The PEPFOLD model1 for all Nef peptides was found to be as the best model with the more negative sOPEP (Optimized Potential for Efficient Structure Prediction) energy. The final selected Nef

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peptide models had sOPEP energy ranging from -34.003 to -15.9598. In addition, the template modeling (TM) values of the Nef peptides models ranged from 0.186 to 0.642 (Table 2). TM scores above 0.5 to 1 were considered perfect, and scores below 0.17 were considered generally weak. The Z-score and Ramachandran plot statics of all optimized Nef peptide models were within an acceptable region (Table 2).

Table 2. Outputs of the structural models of the Nef peptides from PEP-FOLD3.5, PROCHECK and ProSA web servers.

Nef-Peptides	sOPEP	TM Score	Residues in most favored regions (%)	Residues in Additional allowed regions (%)	Residues in disallowed regions (%)	Z-score
Nef-Peptide-1	23.8396	0.377	86/7	13/3	0	-0.53
Nef-Peptide-2	25.4893	0.528	86/7	13/3	0	-1/40
Nef-Peptide-3	21.7697	0.449	86/7	13/3	0	-1/35
Nef-Peptide-4	18.1814	0.384	82/4	17/6	0	-1/16
Nef-Peptide-5	24.3305	0.520	100	0	0	-0/9
Nef-Peptide-6	31.9092	0.619	100	0	0	-1/03
Nef-Peptide-7	15.9598	0.273	92/3	7/7	0	-0/1
Nef-Peptide-8	31.7345	0.407	86/7	13/3	0	-1/03
Nef-Peptide-9	24.426	0.617	86/7	13/3	0	-0/81
Nef-Peptide-10	34.003	0.599	88/2	11/8	0	-1/75
Nef-Peptide-11	26.1485	0.217	92/9	7/1	0	-0/1
Nef-Peptide-12	25.4436	0.186	50	50	0	-0/63
Nef-Peptide-13	22.6902	0.226	85/7	14/3	0	-0/34
Nef-Peptide-14	26.5359	0.428	93/3	6/7	0	-1/37
Nef-Peptide-15	20.6712	0.378	81/2	18/8	0	-2/4
Nef-Peptide-16	16.8204	0.317	66/7	33/3	0	-1/81
Nef-Peptide-17	27.9416	0.340	70/6	29/4	0	-0/39
Nef-Peptide-18	30.7537	0.555	88/2	11/8	0	-0/96
Nef-Peptide-19	20.4103	0.642	91/7	8/3	0	-1/61

According to PROCHECK results, 91.7% of Nef peptide-19 residues were in the most preferred region, 8.3% were in additional allowed regions, and no residues were in disallowed regions. Cross-validation by ProSA also indicated that all PEPFOLD models had a Z-score in the negative range and within the range of experimentally validated proteins (Table 2); therefore, they were considered accurate (27, 28).

The Ramachandran and Z-score plots of the Nef-Peptide-19 model are illustrated in Figures 2a and 2b, respectively. Nef peptide-19 has a Z-score of -1.61 (within the experimentally validated regions), 11 amino acid residues in the preferred regions and none in the disallowed regions.

AMP Prediction

The antimicrobial activity of all Nef peptides was predicted using three AMP predictor web servers: AntiBP2, ClassAMP, and Dbaasps. These web servers utilize full residue analysis to predict antimicrobial properties using machine learning algorithms. The results are annotated in terms of antibacterial, antiviral and antifungal activities. However, the present study focused on antibacterial activity. Based on the results,

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except Nef peptide-3, Nef peptide-9, Nef peptide-14, and Nef peptide-15, all other Nef peptides were predicted to be AMPs by at least one of the servers (Table 3).

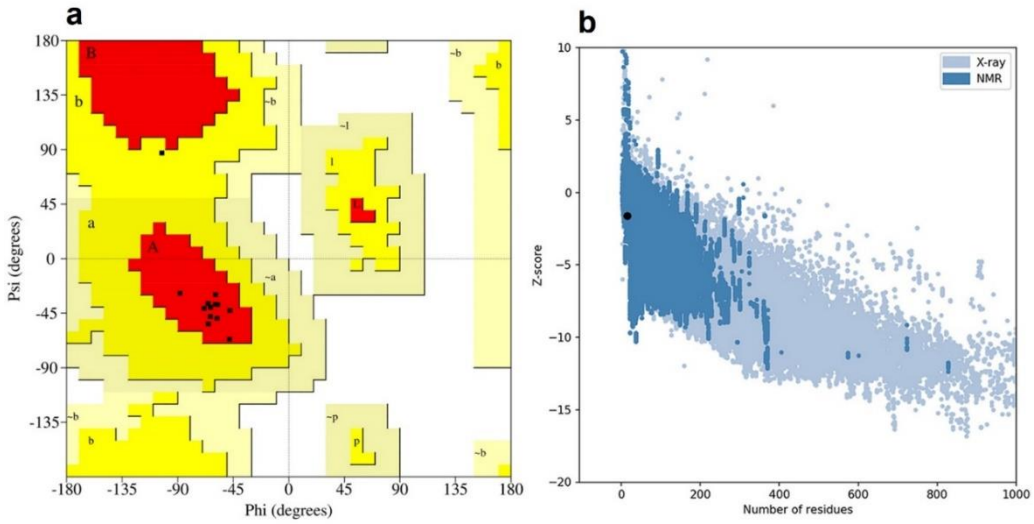

Fig. 2. a) Ramachandran plot for Nef-Peptide-19. Nef-Peptide-19 has 11 amino acid residues in the preferred regions and none in the disallowed regions. b) Nef-Peptide-19 has a z-score of −1.61 and is within the experimentally validated regions.

Table 3. Antimicrobial prediction results using AntiBP2, ClassAMP and Dbaasps web servers.

Nef-Peptides	AntiBP2	ClassAMP (RF)	ClassAMP (SVM)	Dbaasps	Properties
Nef-Peptide-1	No	Antibacterial	Antifungal	Yes	Antimicrobial activities
Nef-Peptide-2	No	Antibacterial	Antiviral	No	
Nef-Peptide-3	No	Antifungal	Antifungal	No	
Nef-Peptide-4	No	Antibacterial	Antiviral	No	
Nef-Peptide-5	Antibacterial	Antibacterial	Antiviral	No	
Nef-Peptide-6	No	Antibacterial	Antifungal	No	
Nef-Peptide-7	No	Antibacterial	Antifungal	No	
Nef-Peptide-8	Antibacterial	Antibacterial	Antifungal	No	
Nef-Peptide-9	No	Antifungal	Antifungal	No	
Nef-Peptide-10	No	Antibacterial	Antiviral	No	
Nef-Peptide-11	No	Antibacterial	Antifungal	No	
Nef-Peptide-12	No	Antibacterial	Antiviral	No	
Nef-Peptide-13	No	Antibacterial	Antifungal	No	
Nef-Peptide-14	No	Antifungal	Antifungal	No	
Nef-Peptide-15	No	Antiviral	Antifungal	No	
Nef-Peptide-16	No	Antibacterial	Antibacterial	No	
Nef-Peptide-17	No	Antibacterial	Antifungal	No	
Nef-Peptide-18	No	Antibacterial	Antifungal	No	
Nef-Peptide-19	No	Antibacterial	Antifungal	No	

Note; Yes means AMP, and No implies non-AMP.

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Nef-Peptide Physiochemical Properties

The prediction of the physicochemical properties of the Nef peptides showed different values for the net charge (from -5 to +4 for Nef-peptide-6 and Nef-peptide-1, respectively), the hydropathicity index (between -1.13 and 0.24) and the amphipathicity index (higher than 0.8 for Nef peptide-1, Nef peptide-9, Nef peptide-14, Nef peptide-18 and Nef peptide-19) (43) (Table 4).

Table 4. Physicochemical properties of the Nef peptides predicted with the dPABBs web server.

Nef-Peptides	Hydropathicity	Amphiphilicity	Net Hydrogen	Charge	pI	MW
Nef-Peptide-1	-0.90	0.80	1.15	4.00	11.73	2303.96
Nef-Peptide-2	-0.94	0.62	1.05	1.00	9.80	2211.76
Nef-Peptide-3	-0.84	0.63	0.80	-0.50	5.50	2007.42
Nef-Peptide-4	-0.48	0.44	0.85	0.50	7.10	2001.41
Nef-Peptide-5	0.24	0.13	0.65	-1.00	4.00	1994.43
Nef-Peptide-6	-0.17	0.38	0.55	-5.00	3.52	2193.65
Nef-Peptide-7	-0.52	0.44	0.65	-3.00	4.10	2318.92
Nef-Peptide-8	0.14	0.75	0.55	0.50	7.10	2125.81
Nef-Peptide-9	-1.13	0.94	1.10	0.50	7.11	2292.90
Nef-Peptide-10	-0.94	0.58	1.15	1.00	7.25	2577.17
Nef-Peptide-11	-0.94	0.20	0.75	-0.50	5.09	2484.03
Nef-Peptide-12	-0.88	0.18	0.75	0.00	6.18	2351.90
Nef-Peptide-13	0.02	0.37	0.60	0.00	6.38	2340.03
Nef-Peptide-14	-1.02	0.80	0.80	-2.00	4.59	2261.82
Nef-Peptide-15	-0.72	0.70	0.80	0.00	6.05	2202.76
Nef-Peptide-16	-0.27	0.39	0.65	-2.00	4.73	2245.84
Nef-Peptide-17	-0.85	0.56	1.00	-3.00	4.30	2469.00
Nef-Peptide-18	-0.97	0.78	1.05	0.50	6.31	2533.09
Nef-Peptide-19	-1.09	0.87	0.93	1.50	7.31	1880.33

Prediction of the secondary structure of Nef peptides using YASARA software indicated that 86.7% of the conformation of Nef peptide19 was α-helix (Table 5). The α-helix structure is the preferred conformation in AMPs and is better able to fit into the membrane (44).

Molecular Docking Analysis

The results of the molecular docking study for the two target proteins MexB and PqsR with the 19 Nef peptides are presented in Tables 7 and 8, respectively. The HADDOCK results of the MexB protein with the 19 Nef peptides showed that Nef-Peptide-19 has the highest HADDOCK score of −136.1, with a Z-score of −1.6 and an RMSD of 0.6 at the DBP active site (Table 6). HADDOCK grouped 128 structures into 13 clusters, describing 64 % of the generated water-refined models.

The HADDOCK results of PqsR with Nef peptides demonstrated that Nef peptide-19 yielded the highest HADDOCK score of -129.4 ± 2.9, with a Z-score of −1.3 and an RMSD of 0.3 at the active site of PqsR's (Table 7). HADDOCK clustered 185 structures in 4 clusters, corresponding to 92% of the generated water-refined HADDOCK models.

Peptide-Protein Complex Interaction Analysis

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Table 5. Content of the secondary structure with the YASARA software. 86.7% of the Nef peptide-19 structure is α-helix.

Nef-Peptides	α-Helix (%)	Random Coil (%)	β Turn (%)	β Sheet (%)
Nef-Peptide-1	35	65	00	00
Nef-Peptide-2	65	35	00	00
Nef-Peptide-3	45	35	20	00
Nef-Peptide-4	40	60	00	00
Nef-Peptide-5	65	35	00	00
Nef-Peptide-6	80	20	00	00
Nef-Peptide-7	30	50	20	00
Nef-Peptide-8	60	20	20	00
Nef-Peptide-9	65	35	00	00
Nef-Peptide-10	75	25	00	00
Nef-Peptide-11	00	60	40	00
Nef-Peptide-12	00	80	20	00
Nef-Peptide-13	00	80	20	00
Nef-Peptide-14	50	50	00	00
Nef-Peptide-15	55	25	20	00
Nef-Peptide-16	30	50	20	00
Nef-Peptide-17	40	40	20	00
Nef-Peptide-18	70	30	00	00
Nef-Peptide-19	86.7	13.3	00	00

Table 6. Nef peptides HADDOCK 2.4 results interacting with MexB protein of *P. aeruginosa*.

Nef-Peptides	HADDOCK score	CLUSTER SIZE	RMSD	VW energy	Electrostatic energy	Desolvation energy	Restraints violation energy	BSA	Z-Score
Nef-Peptide-1	-76.0 ± 1.5	16	5.9 ± 0.1	-56.5 ± 4.3	-183.5 ± 38.6	-7.7 ± 2.5	248.4 ± 48.7	1756.5 ± 29.4	-1.8
Nef-Peptide-2	-82.8 ± 7.1	8	0.3 ± 0.2	-57.4 ± 4.4	-227.8 ± 33.6	4.4 ± 1.6	158.6 ± 48.6	1820.0 ± 41.3	-2.6
Nef-Peptide-3	-72.5 ± 6.1	16	4.4 ± 0.0	-47.9 ± 2.8	-206.8 ± 29.7	-5.5 ± 2.1	222.0 ± 11.2	1645.3 ± 30.9	-2.0
Nef-Peptide-4	-70.6 ± 5.0	19	4.5 ± 0.1	-48.6 ± 7.9	-253.3 ± 50.0	10.0 ± 3.5	186.2 ± 54.4	1673.5 ± 39.5	-2.0
Nef-Peptide-5	-77/3	12	1/0± 0/1	-63/2 ± 5/0	-97/8 ± 4/5	-10/7 ± 1/5	161/4 ± 32/2	1607/8 ± 105/2	-1/6
Nef-Peptide-6	-104.2 ± 6.4	14	0.6 ± 0.0	-66.1 ± 2.6	-210.7 ± 34.6	-11.2 ± 2.8	152.2 ± 10.8	2134.5 ± 43.8	-2.2
Nef-Peptide-7	-89.7 ± 4.2	19	1.5 ± 0.1	-64.1 ± 3.9	-259.1 ± 5.2	1.4 ± 3.2	248.8 ± 78.3	1996.1 ± 52.5	-1.8

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Nef-Peptide-8	-83.9 ± 5.1	31	0.3 ± 0.2	-63.9 ± 6.0	-187.9 ± 35.6	-2.9 ± 2.5	205.4 ± 43.5	1762.8 ± 86.0	-2.2
Nef-Peptide-9	-83.5 ± 6.4	7	6.1 ± 0.1	-57.5 ± 3.0	-292.5 ± 58.5	14.4 ± 4.6	181.1 ± 39.5	2005.4 ± 125.2	-2.0
Nef-Peptide-10	-93.4 ± 5.2	18	0.9 ± 0.0	-59.7 ± 6.2	-237.2 ± 37.4	-12.8 ± 0.6	266.2 ± 43.2	1848.9 ± 46.7	-1.3
Nef-Peptide-11	-92.6± 9.4	10	0.3 ± 0.2	-78.2 ± 3.3	-105.5 ± 17.9	-28.2 ± 6.6	348.9 ± 49.0	2027.1 ± 63.8	-1.9
Nef-Peptide-12	-83.9 ± 1.2	8	1.5 ± 0.0	-74.5 ± 4.0	-102.8 ± 11.2	-8.6 ± 0.9	197.0 ± 60.2	1737.7 ± 18.6	-1.8
Nef-Peptide-13	-96.1 ± 10.2	27	0.3 ± 0.2	-76.6 ± 2.4	-93.9 ± 14.8	-25.3 ± 3.1	245.6 ± 80.3	1870.5 ± 31.5	-2.0
Nef-Peptide-14	-111.8 ± 2.6	12	0.5 ± 0.3	-76.3 ± 2.6	-279.9 ± 27.4	6.7 ± 1.2	138.1 ± 23.1	2174.1 ± 28.1	-2.8
Nef-Peptide-15	-76.6 ± 1.6	27	0.8 ± 0.2	-64.1 ± 2.3	-109.1 ± 17.4	-9.6 ± 1.3	189.8 ± 45.2	1970.6 ± 122.8	-1.7
Nef-Peptide-16	-87.0 ± 5.5	11	0.3 ± 0.2	-60.9 ± 6.9	-203.6 ± 50.3	-11.9 ± 3.4	265.4 ± 63.8	1806.6 ± 99.3	-1.3
Nef-Peptide-17	-102.0± 4.9	17	1.8 ± 0.0	-83.1 ± 3.6	-153.1 ± 32.1	-7.8 ± 1.7	195.5 ± 20.7	2035.1 ± 49.9	-1.6
Nef-Peptide-18	-124.5± 3.4	33	0.3 ± 0.2	-61.0 ± 4.5	-335.9 ± 42.0	-12.3 ± 5.2	159.9 ± 51.4	2085.7 ± 31.7	-2.6
Nef-Peptide-19	-136.1± 1.7	20	0.6 ± 0.3	-74.9 ± 4.2	-258.5 ± 27.9	-9.5 ± 1.0	189.6 ± 58.4	1668.6 ± 61.0	-1.9

Note, RMSD: Root Mean Square Deviation, VW energy: Van der Waals energy, BSA: Buried Surface Area

Table 7. Nef peptides HADDOCK 2.4 results interacting with the PqsR protein of *P. aeruginosa.*

Nef-Peptides	HADDOCK score	Cluster size	RMSD	VW energy	Electrostatic energy	Desolvation energy	Restraints violation energy	BSA	Z-Score
Nef-Peptide-1	-91.6 ± 11.3	8	0.7 ± 0.4	-49.5 ± 8.3	-117.8 ± 30.8	-25.2 ± 1.6	67.3 ± 17.5	67.3 ± 17.5	-1.6
Nef-Peptide-2	-78.9 ± 3.9	101	0.6 ± 0.4	-31.8 ± 3.1	-223.4 ± 28.6	-10.6 ± 2.1	82.1 ± 27.7	1344.2 ± 50.2	-1.3
Nef-Peptide-3	-80.3 ± 1.8	95	0.2 ± 0.1	-53.2 ± 3.8	-106.8 ± 4.8	-14.4 ± 1.3	86.0 ± 31.0	1662.8 ± 51.1	-2.2
Nef-Peptide-4	-86.2 ± 3.5	42	0.4 ± 0.2	-41.6 ± 1.8	-265.6 ± 16.2	0.0 ± 1.7	84.5 ± 24.3	1486.7 ± 66.6	-2.7
Nef-Peptide-5	-79.0 ± 0.9	56	3.4 ± 0.3	-59.0 ± 5.0	-87.2 ± 13.4	-14.3 ± 3.0	116.9 ± 5.6	1559.6 ± 86.0	-1.8
Nef-Peptide-6	-79.0 ± 0.9	56	3.4 ± 0.3	-59.0 ± 5.0	-87.2 ± 13.4	-14.3 ± 3.0	116.9 ± 5.6	1559.6 ± 86.0	-1.8
Nef-Peptide-7	-71.6 ± 2.8	6	2.7 ± 0.0	-42.5 ± 3.1	-144.0 ± 23.1	-10.9 ± 1.2	105.7 ± 22.2	1534.8 ± 34.3	-1.8

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Nef-Peptide-8	-72.5 ± 3.8	16	0.9 ± 0.3	-31.4 ± 3.2	-211.8 ± 40.5	-5.5 ± 4.3	67.9 ± 17.4	1344.9 ± 41.4	-1.8
Nef-Peptide-9	-78.1 ± 3.5	33	0.8 ± 0.5	-32.1 ± 5.6	-304.3 ± 21.6	3.4 ± 1.6	115.3 ± 24.2	1566.8 ± 84.9	-1.2
Nef-Peptide-10	-123.4 ± 7.5	109	0.3 ± 0.2	-58.6 ± 3.4	-191.1 ± 9.9	-35.9 ± 2.3	92.6 ± 24.9	1695.9 ± 69.4	-2.1
Nef-Peptide-11	-95.4 ± 3.2	48	0.3 ± 0.2	-53.1 ± 4.0	-110.8 ± 14.6	-27.9 ± 2.1	77.7 ± 21.1	1618.4 ± 27.8	-2.1
Nef-Peptide-12	-100.2 ± 5.1	52	0.4 ± 0.2	-65.0 ± 3.9	-78.8 ± 17.9	-24.9 ± 3.1	54.7 ± 29.8	1652.7 ± 87.0	-2.3
Nef-Peptide-13	-101.2 ± 2.2	10	0.6 ± 0.4	-58.6 ± 7.5	-120.1 ± 43.3	-27.9 ± 2.2	94.0 ± 15.4	1742.5 ± 78.1	-1.6
Nef-Peptide-14	-67.0 ± 7.6	23	0.9 ± 0.8	-49.2 ± 2.7	-107.5 ± 38.7	-6.2 ± 3.1	99.2 ± 30.4	1457.3 ± 49.7	-1.6
Nef-Peptide-15	-90.5 ± 1.5	75	0.3 ± 0.2	-45.7 ± 1.4	-216.9 ± 18.4	-13.8 ± 1.3	123.5 ± 25.0	1606.9 ± 72.2	-1.8
Nef-Peptide-16	-96.6 ± 7.6	34	0.4 ± 0.3	-50.9 ± 4.0	-212.5 ± 22.1	-9.0 ± 0.8	59.0 ± 12.8	1716.9 ± 27.9	-2.3
Nef-Peptide-17	-83.9 ± 4.6	16	0.4 ± 0.2	-41.8 ± 3.2	-195.7 ± 7.0	-9.7 ± 1.7	67.7 ± 31.5	1548.7 ± 34.3	-1.7
Nef-Peptide-18	-112.5± 2.1	95	0.3 ± 0.2	-59.5 ± 7.8	-134.7 ± 29.5	-33.9 ± 3.9	79.0 ± 28.1	1778.8 ± 24.6	-2.5
Nef-Peptide-19	-129.4 ± 2.9	62	0.3 ± 0.3	-46.6 ± 7.3	-280.7 ± 15.9	-25.8 ± 3.4	17.9 ± 16.4	1486.2 ± 35.5	-1.3

The complex analysis of MexB protein with Nef peptide-19 indicated that 24 residues of MexB binding site interacted with 13 residues of Nef peptide-19. The PDBsum results revealed two ionic, ten hydrogenic and 117 van der Waals interactions in the peptide-protein complex (Figure 3a). The 3D interactions of the MexB binding pocket were illustrated in Discovery Studio (Figure 3b). Among these amino acids, R128, D174, Q176, S180 and D760 of the active site of vital MexB bonded with the residues of Nef peptide-19. Furthermore, H2 and V3 of Nef peptide-19 were in contact with 6 and 4 residues of the active site of MexB, respectively (Figure 3c). Therefore, these two residues of Nef peptide-19 are essential for suppressing MexB protein.

Complex analysis of the active sites of Nef peptide-19 and MexB revealed 129 interactions: two ionic, ten hydrogenic and 117 van der Waals interactions. The critical residues of the active site of MexB (R128, D174, Q176, S180, D760) were involved in hydrogen bonds with Nef peptide-19 residues.

Docking analysis of the PqsR protein and Nef peptide-19 showed that 12 residues of Nef peptide-19 were bound to the 19 residues of the PqsR active site through 120 interactions (two were ionically bound, 10 were hydrogen bonded, and 108 were not bound) (Figure 4a).

The 3D interactions of the PqsR binding pocket were visualized in Discovery Studio (Figure 4b). Among these residues, seven of the essential amino acids of the active site of PqsR, I149, I186, L189, L207, I236, Y258, and T265 (45), were suppressed by the residues of Nef peptide-19 (Figure 4c).

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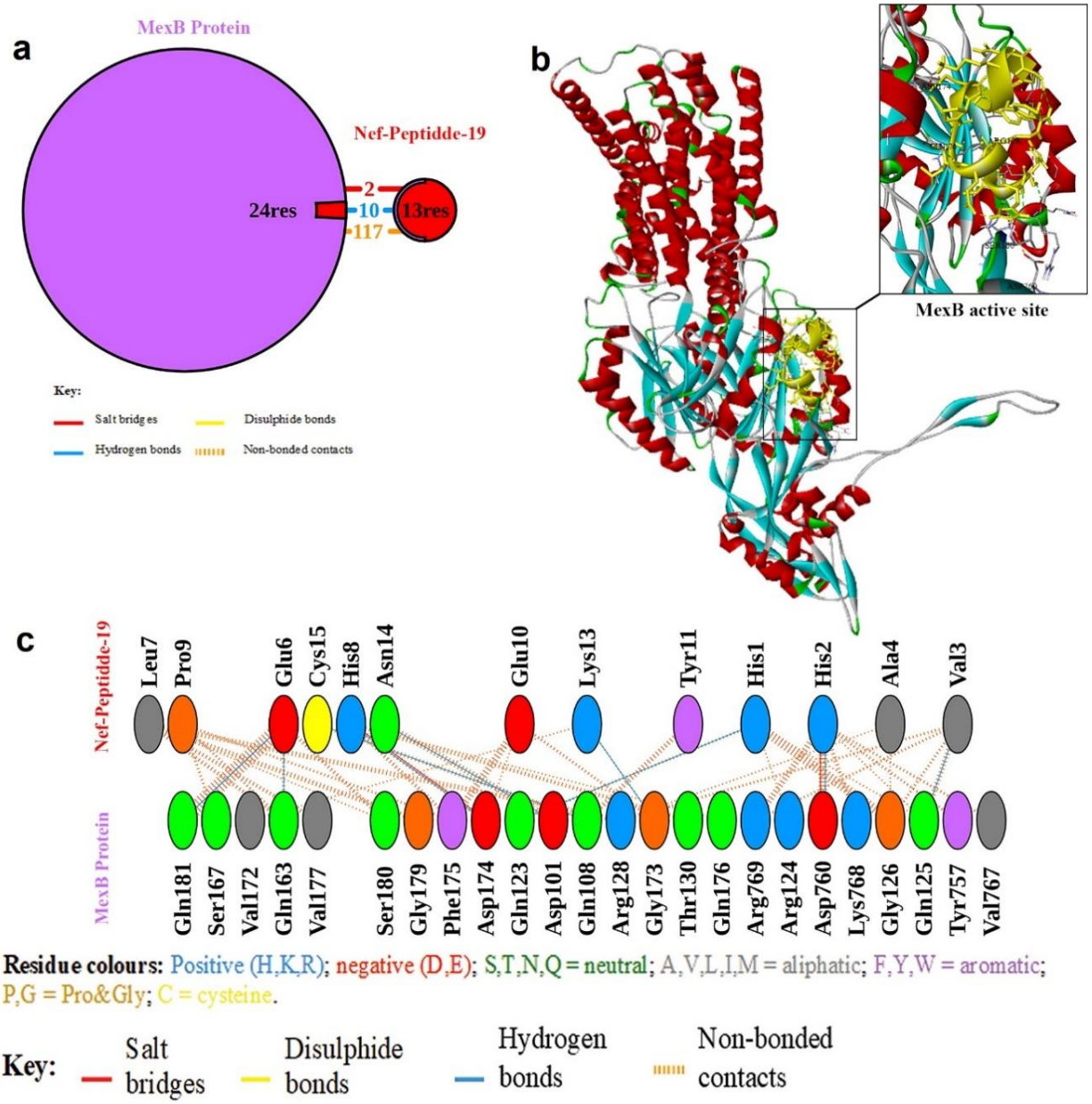

Fig. 3. Analysis of the MexB_Nef peptide-19 complex. **a** Schematic representation of the MexB binding pocket and the Nef peptide-19 complex. **b** Crystal structure of MexB (PDB: 6IIA) bound to Nef-Peptide-19 (yellow). **c** 2D diagram of the interactions between Nef peptide-19 and MexB.

Analysis of *in vitro* evaluations

Assessment of Antibacterial Activity

The results of antibacterial evaluation of Nef peptides against *P. aeruginosa* (PTCC 1074) revealed that six Nef peptides had significant MIC values, as shown in Table 8. Among them, Nef-Peptide-19 was

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the most effective peptide, with MIC and MBC values of 10 µM (18.8 µg/mL) and 20 µM (37.6 µg/mL), respectively.

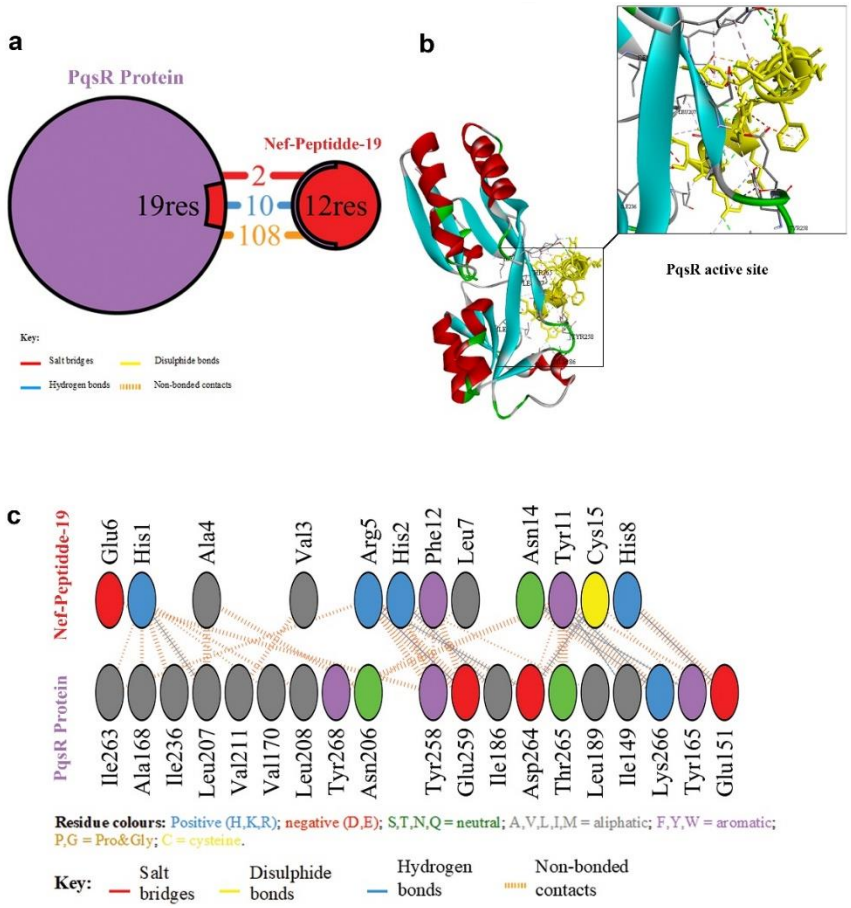

Fig. 4. Analysis of the interactions between PqsR and Nef peptide-19. a) Schematic representation of the PqsR binding site with the Nef peptide-19 complex. b) Crystal structure of PqsR (PDB: 4JVD) bound to Nef peptide-19 (yellow). **c** 2D diagram of the Nef peptide-19 and PqsR interactionsL.

Table 8. Antibacterial activities of the best Nef peptides by MIC test.

Nef-Peptides	MIC values (µM)
Nef-Peptide-1	20 (37.6 µg/mL)
Nef-Peptide-10	20 (37.6 µg/mL)
Nef-Peptide-14	20 (37.6 µg/mL)
Nef-Peptide-16	20 (37.6 µg/mL)
Nef-Peptide-18	20 (37.6 µg/mL)
Nef-Peptide-19	10 (18.8 µg/mL)

Assessment of Antibiofilm Activity

Antibiofilm evaluation of the top six Nef peptides against *P. aeruginosa* (PTCC 1074) showed that Nef peptide-19 was the most active peptide. The percentage inhibitory effect of Nef peptide-19 on biofilm formation was 56.23 at the concentration of 20 µM (37.6 µg/mL). All antibiofilm activities of Nef peptides are demonstrated in Figure 5.

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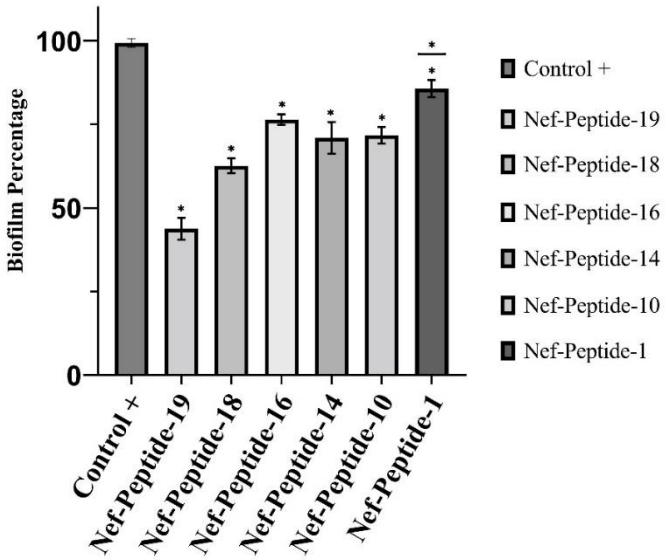

Fig. 5. The percentage of biofilm formation of P.aeruginosa in the presence of the top six Nef peptides at 20 µM. Values were presented as mean and standard deviation (±SD) from triplicate independent assays (two-way ANOVA, P-value<0.05).

Discussion

The increase in MDR bacteria has led to a decrease in the efficacy of conventional antibiotics in recent years (46). *P. aeruginosa* is already resistant to various antibiotics, including ciprofloxacin, gentamicin, imipenem, fluoroquinolone, and ceftazidime (47). The use of AMPs instead of conventional antibiotics has been considered a new strategy to combat MDR bacteria (48). Researchers have recently focused on viral-derived peptides to assess their antimicrobial activity. For instance, pepR (isolated from the dengue virus capsid protein) is active against *Staphylococcus aureus* biofilms (49), M2 AH (isolated from the influenza virus M2 protein) is active against influenza virus infections (50), and P24-derived peptides (isolated from the HIV-1 virus P24 protein) are active against the HIV-1 virus (51).

New AMPs can be developed by using evolved prediction tools that search the protein sequences of different organisms (52). Some peptides derived from HIV-1 proteins, such as gp41, gp120 and p24, have been investigated for their potential as antimicrobial agents (51, 53). In the current study, bioinformatics and laboratory techniques were used to assess the antimicrobial activities of 19 peptides from the HIV-1 Nef protein against *P. aeruginosa*. To our knowledge, HIV-1 Nef peptides had moderate to strong antibacterial activities against *P. aeruginosa*.

Moreover, in the present study, 19 Nef peptides with two pathogenic proteins (MexB and PqsR) were docked via HADDOCK. The 2021 study by Dong et al. on the MexB protein revealed that targeting MexB with the BING peptide (isolated from *Oryzias latipes*) sensitizes MDR bacteria, including *P. aeruginosa*, to antibiotics (54). The molecular docking results of the current study showed that Nef peptide-19 fitted into the binding site MexB via several residues, including two crucial residues in DBP, R128 and S180 (10, 55) with a HADDOCK score of -136.1 ± 1.7. Compounds with a molecular mass greater than 1000 (such as Nef Peptide-19) can bind to the DBP of MexB and disrupt EPS function (55). PqsR is another pathogenic

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protein of *P. aeruginosa* that has been described in previous studies as a potential target for disrupting the QS system and reducing the expression of several virulence factors (56, 57). Our docking analysis revealed that Nef peptide-19 has the highest affinity for the PqsR binding site and contacts key residues such as I149, I186, L189, L207, L208, I236, Y258 and T268 (45, 58) with a HADDOCK score of -129.4 ± 2.9.

Several AMPs have been studied *in vitro* to suppress *P. aeruginosa* infections (59, 60). In 2020, Li et al. reported the antibacterial activity of CM4, a cationic AMP from the silkworm *Bombyx mori*, against *P. aeruginosa* with an MIC value of 18 µM (61). 6K-F17, a peptide synthesized by Beaudoin et al. in 2018, showed MIC values of 2-256 µg/ml against several species of *P. aeruginosa* (62). Compared to the reported results, Nef peptide-19 showed considerable bacteriostatic and bactericidal activities against *P. aeruginosa* with MIC and MBC values of 10 µM (18.8 µg/ml) and 20 µM (37.6 µg/ml), respectively. In 2022, Aflakian et al. suggested that three novel synthesized AMPs (WSF, FASK, YDVD) at a concentration of 800 µg/ml could inhibit the biofilm production of *P. aeruginosa* (63). Furthermore, Gopal et al. reported in 2013 that NRC-16 affects the production of *P. aeruginosa* from 2.17 to 17.4 µg/ml (64). The results of the ongoing study revealed that Nef peptide-19 at a concentration of 20 µM (37.6 µg/ml) was able to inhibit 53.23% of biofilm formation. Based on these results, we hypothesize that Nef peptide-19 could be a potential new AMP to fight bacterial infections.

In order to advance the development of Nef peptides as potential therapeutics against P. aeruginosa, certain limitations need to be addressed in future research. These could include evaluating the efficacy of Nef peptides against a broader range of clinical isolates, assessing their antibacterial and antibiofilm activities using an in vivo model, and testing the stability of the AMPs under physiological conditions to ensure their safety.

Acknowledgments

The authors would like to thank the University of Isfahan for supporting this study.

Ethical considerations

This research was financially supported by the University of Isfahan [grant number UI14010310]. This article does not include studies with human participants or animals conducted by any of the authors.

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[DOI: 10.22088/IJMCM.BUMS.13.1.46]