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RESEARCH ARTICLE

Effect of secondary metabolites from *Streptomyces* sp. KSF103 on dengue virus 2 replication competency in C6/36 cells and identification of potent dengue virus 2 inhibitors

Amelia-Yap, Z.H.[1], Low, V.L.[1*], Azman, A.S.[2], Sam, S.S.[1], Teoh, B.T.[1], Vinnie-Siow, W.Y.[1], Tan, T.K.[3], Hassandarvish, P.[1*]

[1]Tropical Infectious Diseases Research and Education Centre (TIDREC), Higher Institution Centre of Excellence, Universiti Malaya, 50603, Kuala Lumpur, Malaysia
[2]School of Science, Monash University Malaysia, Jalan Lagoon Selatan, 47500, Bandar Sunway, Malaysia
[3]Department of Parasitology, Faculty of Medicine, Universiti Malaya, 50603, Kuala Lumpur, Malaysia
*Corresponding authors: vanlun_low@um.edu.my (Low, V.L.), pouyahassandarvish@um.edu.my (Hassandarvish, P.)

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ABSTRACT

Streptomyces sp. KSF103 has demonstrated promising insecticidal properties, prompting a subsequent study to evaluate the *in vitro* effects of its ethyl acetate (EA) extract on dengue virus type 2 (DENV-2) replication in *Aedes albopictus* C6/36 cells. Pre- and post-treatment assays revealed significant inhibition of viral replication, indicating that the EA extract disrupts both viral entry and adsorption in pre-treated cells, as well as intracellular replication in post-treated cells. Chemical profiling using liquid chromatography–mass spectrometry (LC-MS) identified several bioactive compounds in the extract, including pentanamide, C17 sphinganine, dichamanetin, dodemorph, and antillatoxin B. Further *in silico* molecular docking analysis targeting DENV-2 NS2B/NS3 protease, NS5 polymerase, and envelope (E) protein revealed that antillatoxin B and dichamanetin exhibit strong binding affinities, supporting their potential antiviral activity. These findings align with the observed inhibitory effects of the EA extract and highlight its potential as a source of potent DENV-2 inhibitors.

Keywords: Actinobacteria; antivirus; dengue; infectious diseases; natural products.

INTRODUCTION

Streptomyces sp. KSF103 has garnered attention due to its potent insecticidal activity against mosquitoes. Recent findings suggest that the ethyl acetate (EA) extract of *Streptomyces* sp. KSF103 may exert its insecticidal effects by disrupting energy metabolism and interfering with key metabolic enzymes, particularly fructose-bisphosphate aldolase (FBA), a critical enzyme in the glycolytic pathway (Tan *et al*., 2023). Another study has also reported its ability to induce apoptosis and inhibit acetylcholinesterase activity, highlighting its potential as a novel biocontrol agent (Amelia-Yap *et al*., 2024). In parallel, the methanolic extract of *Streptomyces* sp. KSF103 has also demonstrated antiviral activity against dengue virus serotype 2 (DENV-2) (Zulkifli *et al*., 2023), suggesting a dual mode of action that targets both the vector and the pathogen. However, the mechanism behind its antiviral effect, especially in the context of mosquito vector systems, remains to be elucidated.

Accordingly, this study was conducted to explore the potential of *Streptomyces* sp. EA secondary metabolites in inhibiting DENV-2 replication competency in *Aedes albopictus* C6/36 cell lines. The chemical constituents of the EA extract were profiled using liquid chromatography–mass spectrometry (LC-MS), and potential DENV-2 inhibitors were identified through *in silico* molecular docking studies.

MATERIALS AND METHODS

Biosafety and biosecurity approval

Approval for the use of DENV-2 in this study was obtained from the Institutional Biosafety and Biosecurity Committee (IBBC) of Universiti Malaya (UMIBBC/NOI/R/TNCPNI/TIDREC-011/2021).

Cell lines

The *Aedes albopictus* mosquito cell line (C6/36 cells) and the African green monkey kidney cell line (Vero cells) were obtained from the American Type Culture Collection (ATCC, Virginia, USA). C6/36 cells were cultured and maintained in Eagle's Minimum Essential Medium (EMEM, Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS). The cells were incubated at 28°C in 5% CO_2 and were sub-cultured or cryopreserved when they were 80% confluent.

Vero cells were cultured and maintained in Dulbecco's Minimum Essential Medium (DMEM) (Thermo-Fisher Scientific, MA, USA) containing 10% FBS. The cells were incubated at

37°C with 5% CO_2 supplemented and were subcultured or cryopreserved when they were 80% confluent.

During treatments, culture media supplemented with 2% FBS was used as the maintenance medium for respective cell lines.

Fermentation and extraction of bioactive compound

The fermentation and extraction of bioactive compounds from *Streptomyces* sp. KSF103 in this study was performed as outlined in Amelia *et al*. (2023). Briefly, the fermentation and extraction of bioactive compounds from *Streptomyces* sp. KSF103 in this study followed modified methods described by Ganesan *et al*. and Thenmozhi *et al*. The strain was initially cultured on ISP2 agar and then transferred into ISP2 broth for large-scale fermentation at 28°C for 14 days with agitation. The culture broth was separated from the mycelium by centrifugation, and the supernatant was extracted with ethyl acetate. The organic layers were concentrated using a rotary evaporator at 40°C to obtain the ethyl acetate (EA) extract, which was stored at -20°C.

Dengue virus type 2

DENV-2 was selected for this study as it is one of the most virulent and epidemiologically dominant types of dengue virus, often associated with severe clinical manifestations. The DENV-2 New Guinea C strain (NGC) was purchased from American Type Culture Collection (ATCC, USA). To ensure the continuous supply of viruses throughout all experiments, propagation of DENV-2 was carried out. Virus propagation and the evaluation of DENV-2 replication competency were performed on C6/36 cells, whereas virus titration was performed on Vero cells using foci forming assay (FFA).

A monolayer of C6/36 cells was cultured in a 75 cm^2 flask (Corning, USA) in EMEM supplemented with 2% FBS. Upon achieving 80% confluency, the cells were inoculated with DENV-2 in 2 ml serum-free EMEM. The flask was placed on a rocker for 30 min and then incubated in a 28°C incubator with 5% CO_2 for successful infection. After incubation, the flask medium was topped up to 10 mL using EMEM containing 2% FBS and subsequently proceeded with incubation for 7 days in a 28°C incubator supplemented with 5% CO_2. The infected cells were observed daily for the sign of virus cytopathic effect (CPE). On day 7 when the virus CPE was obvious, the supernatant was harvested and transferred to a Falcon tube. The culture supernatant was centrifuged at 4°C and 3,000 rpm for 10 minutes to remove cell debris. Then, the supernatant containing DENV-2 was aliquoted into 1.5 mL tubes and stored at –80°C until needed.

DENV-2 titration

Virus titration was performed using a focus forming assay (FFU). Virus supernatants were 10-fold serially diluted in DMEM with 2% FBS and added to Vero cell monolayers (1 × 10^5 cells/well) in 24-well plates. Plates were rocked for 30 min, then incubated at 37°C for 1 hour. After incubation, the inoculum was removed and cells were overlaid with 0.7% high-viscosity carboxymethylcellulose (CMC) in 2% FBS-DMEM, followed by 4 days incubation at 37°C with 5% CO_2.

Cells were then washed twice with PBS and fixed with 200 µL/well of 4% paraformaldehyde for 30 min. After three PBS rinses, cells were permeabilised with 1% Igepal (200 µL/well, 15 min, RT). Blocking was performed with 3% skim milk (R&M Chemicals, UK) in PBS. Cells were then incubated with polyclonal dengue-immune human sera for 1 hour at room temperature, followed by HRP-conjugated anti-human IgG (Abcam, UK) for another hour.

After each step, monolayers were washed thrice with PBS. Foci were visualised using 3,3'-Diaminobenzidine (DAB) substrate (Thermo Fisher, USA), and the reaction was stopped by rinsing with distilled water. Foci were enumerated visually, and the virus titer was calculated as:

FFU/mL = (average foci / volume) × dilution factor × (volume / 1 mL).

DENV-2 infections (Pre-treatment)

C6/36 cells (2 × 10^4 cells/well) were seeded in a 96-well plate and incubated overnight in 28°C 5% CO_2 incubator. *Streptomyces* sp. KSF103 EA extract was serially diluted at a range of final concentrations of 14, 7, 3.5, 1.75, and 0.875 µg/mL in EMEM media containing 2% of FBS. Cells were pre-treated with different concentrations of the EA extract and incubated for 24 hour inside a 28°C 5% CO_2 incubator. After 24 hour of incubation, the treatment was removed, and cells were washed with PBS. Pre-treated cells were infected with DENV-2 at multiplicity of infection (MOI) of 1 and incubated at room temperature in the rocking machine (10 rpm) for 15 min, then transferred to an incubator at 28°C with 5% CO_2 for 1 hour. After the incubation, the inoculum was removed. The cells were washed with PBS, and 2% FBS supplemented EMEM media were added and further incubated for 96 hour at 28°C 5% CO_2 incubator. The infected cell culture supernatant was harvested after 96 hour and stored at -80°C for virus titration. Viral RNA copy number was determined using qRT-PCR. Uninfected and virus infected controls (non-drug treated cells) were run in parallel as normal and virus controls, respectively. The experiments were performed in triplicates.

DENV-2 infections (Post-treatment)

C6/36 cells (2 × 10^4/well) were seeded and grown overnight on a 96-well plate. The cells were infected with DENV-2 at MOI of 1. The cells were first incubated at room temperature in the rocking machine (10 rpm) for 15 min, then transferred to the incubator and incubated at 28°C with 5% CO_2 for 1 hour to allow virus attachment and entry into the host cells. The inoculum was removed, and C6/36 cells were washed with PBS to remove unabsorbed viruses. The virus-infected cells were treated with serially diluted concentrations of the extract at 3.5, 1.75, 0.875, and 0.4375 µg/mL prepared in the maintenance medium. After incubation for 96 hour at 28°C, the supernatants were harvested and kept at -80°C. Viral RNA copy number was determined using qRT-PCR. Uninfected and virus-infected controls (non-drug treated cells) were run in parallel as normal and virus controls, respectively. The experiments were performed in triplicate.

Viral RNA extraction and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Viral RNA extraction was performed using the NucleoSpin RNA Virus Mini extraction kit (Macherey-Nagel, Germany) according to the manufacturer's protocols. The primers used for the DENV-2 RNA quantification by qRT-PCR were previously described (Seah *et al*., 1995). A one-step qRT-PCR was carried out in the QuantStudio™ 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, USA) with SensiFAST SYBR Hi-ROX one-step kit (BioLine, UK) following the manufacturer's protocol, and using serially diluted standard samples. A total of 20 µL reaction per sample was prepared, containing 10 µL 2x SensiFAST SYBR® Hi-ROX Mix, 0.5 µL 10 µM forward and reverse primers each, 0.2 µL reverse transcriptase, 0.4 µL RiboSafe RNase Inhibitor, 2 µL of the RNA template, and 6.4 µL DEPC-treated water. The thermal cycling conditions were: initial denaturation at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. The specificity of the amplified product was verified by melting curve analysis. The viral RNA copy numbers were determined based on a standard curve generated with a 6-fold serially diluted viral RNA extracted from the DENV-2 virus stock of a known infectious titer.

Data analyses

The data presented were the mean ± SD of three independent experiments. The student t-test was used to analyse the inhibition property of *Streptomyces* sp. KSF103 EA extract in C6/36 cells against DENV-2 compared to a negative control.

Chemical profiling of *Streptomyces* sp. KSF103 EA extract

The chemical profiling of *Streptomyces* sp. KSF103 EA extract was carried out by LC-MS Platform from Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia. The identification of small molecules or compounds in the EA extract was performed according to the small molecule-Quadrupole Time of Flight (SM-QTOF) guidelines. The identified compounds in the EA extract were matched via the Metlin database based on particular m/z values. To further narrow down the results as multiple compounds can have similar m/z values, compounds present in both positive and negative ion mode were excluded and compounds with Score Db or Score MFG close to 100 were selected. Only compounds with Diff (Db.ppm) or Diff (MFG.ppm) values within -2 to +2 were chosen from the list of compounds.

Receptor and ligand preparation

The three-dimensional structures of the dengue virus NS2B/NS3 (PDB ID: 2FOM), NS5 (PDB ID: 2J7U), and E (PDB ID: 1OKE) proteins were retrieved from the Protein Data Bank (PDB) (http://www.rscb.org). The crystal structures were obtained at 1.5 Å resolution. The protein structures were minimised by applying the CHARMM27 force field. Pentanamide, c17 sphinganine, dichamanetin, dodemorph, and antillatoxin B were used as ligands in this experiment. The structures of the ligands were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and subsequently imported into the Discovery Studio 3.5 software for minimisation prior to the docking process. Before docking with the five ligands, the water molecules and inhibitor ligands were deleted from the receptor proteins. Both the receptors and ligands were converted and saved as PDBQT files using the AutoDock Vina 1.5.6 software.

Molecular docking

In this study, the binding properties of the ligands to each of the target proteins, namely the NS3/NS2B, NS5, and E proteins of DENV-2, were examined using the AutoDock Vina 1.5.6 software. The compounds and proteins of interest were added with hydrogen molecules before saving them as PDBQT files. Blind dockings were carried out to discover the binding affinity of the ligands with different sites of the target receptors. All the receptors were placed into the grid box in AutoDock Vina software to find the ideal demission grid information before molecular docking. Grid boxes of sizes sufficient to cover the whole target receptors were carefully constructed based on the parameters as tabulated in Table 1. The grid boxes were chosen carefully to ensure the whole protein of interest fit in and had close contact with ligands.

Molecular docking analyses

AutoDock Vina 1.5.6 and Discovery Studio 3.5 were used to evaluate docking results. The output PDBQT files were loaded into AutoDock Vina to visualize ligand conformations with each receptor, ranked by binding affinity. Data on hydrogen bonds, pi–pi, pi–cation interactions, and contact residues were recorded. To further analyze interaction energies (van der Waals and electrostatic) and compute binding energy, Discovery Studio 2.5 was used. PyMOL was employed to merge conformations into a single file before analysis in Discovery Studio 3.5. The total of interaction and binding energy, termed complexation energy, was used to identify the most active inhibitors.

RESULTS

Effect of *Streptomyces* sp. KSF103 EA extract against *in vitro* replication competency of DENV-2 on pre-treated C6/36 cells

C6/36 cells pre-treated with *Streptomyces* sp. KSF103 EA extract demonstrated a reduction in DENV-2 yields in a concentration-dependent manner. To determine the concentration-dependent effect of the EA extract on DENV-2 replication, C6/36 cells were first treated with increasing concentrations of the extract and later infected with DENV-2. The results in Figure 1 showed that the EA extract at concentrations of 0.875, 1.75, 3.5, 7, and 14 µg/mL significantly inhibited DENV-2 infection in C6/36 cells ($P < 0.0001$). In this experiment, the half maximal inhibitory concentration (IC_{50}) value of the EA extract determined by qRT-PCR was 1.271 µg/mL (Table 2). The highest inhibition of viral infectivity of DENV-2 by the EA extract was 90.5% when the virus was treated at a concentration of 14 µg/mL (Figure 1).

Table 1. Parameters used for molecular docking of pentanamide, c17 sphinganine, dichamanetin, dodemorph, and antillatoxin B with each target protein. All grid boxes with a spacing size of 1.000 Å have sufficient sizes to cover the entire protein structures during molecular docking

Proteins	Center-X	Center-Y	Center-Z	Size-X	Size-Y	Size-Z
NS3/NS2B	-1.365	-14.308	17.508	50	54	44
NS5	25.206	56.326	17.032	74	74	72
E	-10.095	35.958	-15.288	60	44	118

Table 2. IC_{50} of pre-treatment assay

Compound	CC_{50} (µg/mL)	IC_{50} (µg/mL)
		qRT-PCR
Streptomyces sp. KSF103 EA extract	152.2	1.271
	Selectivity Index (SI) =	119.75

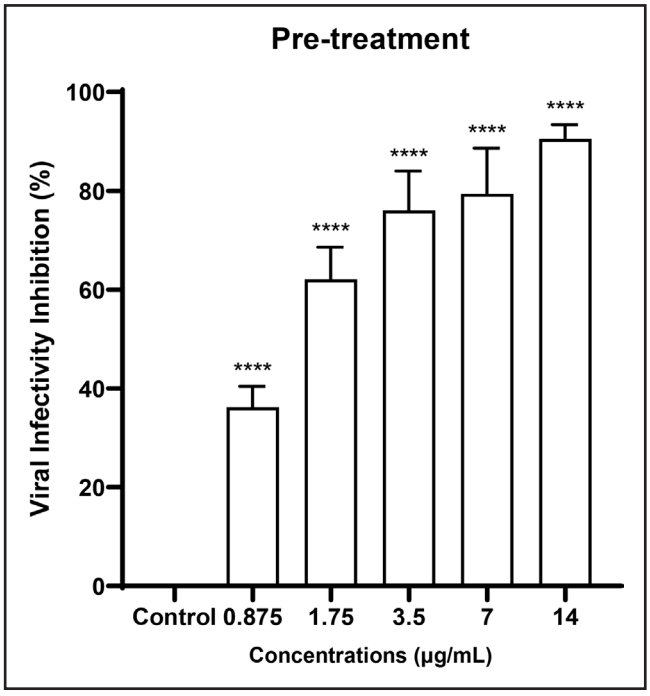

Figure 1. *Streptomyces* sp. KSF103 EA extract exhibited inhibitory effect against DENV-2 in pre-treated C6/36 cells in a concentration-dependent manner. Data presented as mean ± SD. Error bars indicate the range of values obtained from three independent experiments. ****$P < 0.0001$ indicated a significant difference compared to the virus control analysed by the t-test.

Effect of *Streptomyces* sp. KSF103 EA extract against *in vitro* replication competency of DENV-2 on post-treated C6/36 cells

To determine *Streptomyces* sp. KSF103 EA extract in inhibiting DENV-2 post-treatment, C6/36 cells were infected with DENV-2 followed by the treatment with the extract The EA extract exhibited inhibitory effect after C6/36 cells were infected with DENV-2. The post-treatment experiments indicated *Streptomyces* sp. KSF103 EA extract decreased the yields of DENV-2 in C6/36 cells in a concentration-dependent manner. The data in Figure 2 illustrated that the EA extract at concentrations of 0.4375 µg/mL ($P < 0.05$), 0.875 µg/mL ($P < 0.01$), 1.75 µg/mL ($P < 0.0001$), and 3.5 µg/mL ($P < 0.0001$) significantly inhibited DENV-2 infection in C6/36 cells. In this assay, the half maximal inhibitory concentration (IC_{50}) value of the EA extract was determined at 0.9127 µg/mL (Table 3). Viral RNA copy number quantifications indicated that the infectious viral titers of DENV-2 were reduced when the EA extract treatment was added after its infection on C6/36 cells. The highest inhibition of DENV-2 infectivity was 76.47% when 3.5 µg/mL of the EA extract was added to the infected cells (Figure 2).

Chemical profiling via liquid chromatography-mass spectrometry (LC-MS)

A total of 18 compounds were detected from the EA extract through LC-MS determined by post-screening (Table 4). Only 5 compounds were reported with bioactivities, namely pentanamide, c17 sphinganine, dichamanetin, dodemorph and antillatoxin B. Their bioactivities include insecticidal effect, anticancer, mycotoxin, anticancer, antibacterial, fungicide and neurotoxin. The bioactivities of the remaining 13 compounds are yet to be discovered.

Table 3. IC_{50} of post-treatment assay

Compound	CC_{50} (µg/mL)	IC_{50} (µg/mL)
		qRT-PCR
Streptomyces sp. KSF103 EA extract	152.2	0.9127
	Selectivity Index (SI) =	166.76

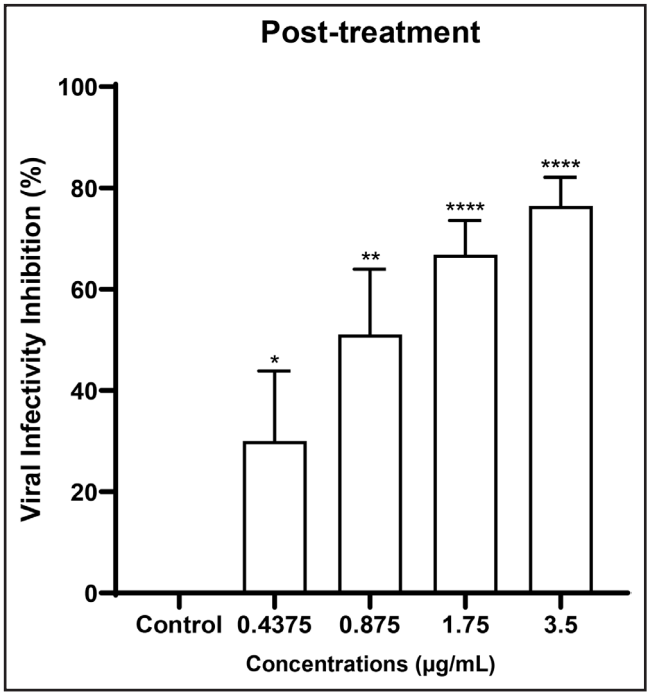

Figure 2. *Streptomyces* sp. KSF103 EA extract exhibited inhibitory effect against DENV-2 in post-treated C6/36 cells in a concentration-dependent manner. Data presented as mean ± SD. Error bars indicate the range of values obtained from three independent experiments. *p < 0.05, **p < 0.01, ****p < 0.0001 indicated a significant difference compared to the negative control analysed by the t-test.

Table 4. LC-MS secondary metabolites identified in *Streptomyces* sp. KSF103 EA extract and their established bioactivities

No	RT (min)	Base peak (m/z)	Proposed compounds	Molecular formula	Mass (g/mol)	Bioactivity	Reference(s)
			Positive ion mode				
1	2.656	102.0914	Pentanamide	$C_5 H_{11} N O$	101.084	Insecticide	Pridgeon *et al*. (2014)
2	12.553	288.2893	C17 Sphinganine	$C_{17} H_{37} N O_2$	287.282	Anticancer, mycotoxin	Du *et al*. (2008); El-Garawani *et al*. (2020)
3	17.814	486.1916	Dichamanetin	$C_{29} H_{24} O_6$	468.1568	Anticancer, antibacteria	Yong *et al*. (2013); Koudokpon *et al*. (2018)
4	19.194	282.2794	Dodemorph	$C_{18} H_{35} N O$	281.272	Fungicide	Vorkamp *et al*. (2003)
5	8.535	438.2716	Lys Tyr Lys	$C_{21} H_{35} N_5 O_5$	437.2642		
6	23.208	338.3416	N-Cyclohexanecarbonylpentadecylamine	$C_{22} H_{43} N O$	337.3342		
7	13.803	316.3215	13-methyl-octadecanoic acid	$C_{19} H_{38} O_2$	298.2875		
8	15.495	366.1697	Erybraedin E	$C_{22} H_{20} O_4$	348.1358		
9	14.377	215.0705	3,4-Dihydroxy-3,4-dihydro-9-fluorenone	$C_{13} H_{10} O_3$	214.0633		
10	16.585	301.1434	4'-Hydroxy-5,7-dimethoxy-8-methylflavan	$C_{18} H_{20} O_4$	300.136		
11	1.781	100.0755	1-Pyrrolidinecarboxaldehyde	$C_5 H_9 N O$	99.0684		
			Negative ion mode				
12	18.408	564.3449	Antillatoxin B	$C_{33} H_{47} N_3 O_5$	565.3519	Neurotoxin	Li *et al*. (2001)
13	8.534	472.2328	Lys Tyr Lys	$C_{21} H_{35} N_5 O_5$	437.2635		
14	9.106	183.0665	2-Hydroxy-6-oxo-7-methylocta-2,4-dienoate	$C_9 H_{12} O_4$	184.0739		
15	7.599	172.0982	Isovalerylalanine	$C_8 H_{15} N O_3$	173.1054		
16	8.973	347.1942	Thr Lys Thr	$C_{14} H_{28} N_4 O_6$	348.2014		
17	8.407	365.1581	His Pro Asn	$C_{15} H_{22} N_6 O_5$	366.1653		
18	9.012	264.1255	6-N,4-diphenyl-1,4-dihydro-1,3,5-triazine-2,6-diamine	$C_{15} H_{15} N_5$	265.1328		

Molecular docking

Pentanamide, c17 sphinganine, dichamectin, dodemorph, antillatoxin B, five compounds isolated from *Streptomyces* sp. KSF103 EA extract, active against three DENV proteins, namely NS3/NS2B, NS5 and E proteins, were subjected for molecular docking evaluations using AutoDock Vina. Autodock Vina ranked the results for interaction based on the affinity energy (Table 5). The interactions of Antillatoxin B and dichamanetin with dengue virus proteins are shown in Figures 3–8. The docking studies show that both antillatoxin B and dichamanetin exhibit the strongest binding affinities with NS3/NS2B, NS5 and E proteins. Antillatoxin B exhibits the strongest binding affinity against NS5 (-8.6 kcal/mol), followed by E protein (-7.7 kcal/mol), and NS3/NS2B (-7.5 kcal/mol). Dichamanetin exhibits the strongest binding affinity against NS5 (-8.4 kcal/mol), followed by NS3/NS2B (-7.9 kcal/mol), and E protein (-7.3 kcal/mol). Only receptors interacted with compounds at less than -7 kcal/mol were shown.

Hydrogen bonding and Pi interaction of antillatoxin B and dichamanetin between the three DENV proteins were shown in Table 6. The Discovery Studio 3.5 showed close amino acid residues found in the binding pocket between antillatoxin B and NS3/NS2B, namely HIS51 and GLY153. Pi-pi interactions with TYR161 were observed. Close residues of NS3/NS2B with dichamanetin were demonstrated at ARG55 and GLY153. Pi-pi interactions were observed at TYR161 and HIS51.

Antillatoxin B and NS5 shows close contact with amino acid residues ASP538, ASP609, ASP663, GLY604, and ASP538. Antillatoxin B exhibits pi-pi interation with TRP477. Hydrogen bonds linked dichamanetin and close contact via the GLY601, ASP663, GLY604 and TYR606 NS5 residues. Dichamanetin exhibits pi-pi interactions with PHE398, TYR606, PHE485, TRP477 and VAL603.

Another essential protein, DENV E protein, shows pi-pi interaction between antillatoxin B and HIS27. Pi-cation interaction was observed at LYS394 residue. The molecular docking results between E protein and dichamanetin showed close contact via the ARG9, THR48, GLN271 and GLN233 residues. Pi-cation interaction was observed at LYS284 residue.

Table 5. The highest binding affinity of each compound with DENV proteins

Compounds	Protein	Affinity (kcal/mol)
Antillatoxin B	NS3/NS2B	-7.5
	NS5	-8.6
	E	-7.7
C17 Sphinganine	NS3/NS2B	-4.3
	NS5	-4.4
	E	-3.4
Dichamanetin	NS3/NS2B	-7.9
	NS5	-8.4
	E	-7.3
Dodemorph	NS3/NS2B	-6.8
	NS5	-6.9
	E	-5.5
Pentanamide	NS3/NS2B	-3.8
	NS5	-3.9
	E	-4.1

Table 6. Hydrogen bonding and pi interactions between the compounds and respective proteins

Compound	Protein	Hydrogen bonding	Pi interaction
Antillatoxin B	NS3/NS2B	HIS51, GLY153	TYR161
	NS5	ASP538, ASP609, ASP663, GLY604, ASP538	TRP477
	E	N/A	HIS27, LYS394
Dichamanetin	NS3/NS2B	ARG55, GLY153	TYR161, HIS51
	NS5	GLY601, ASP663, GLY604, TYR606	PHE398, TYR606, PHE485, TRP477, VAL603
	E	ARG9, THR48, GLN271, GLN233	LYS284

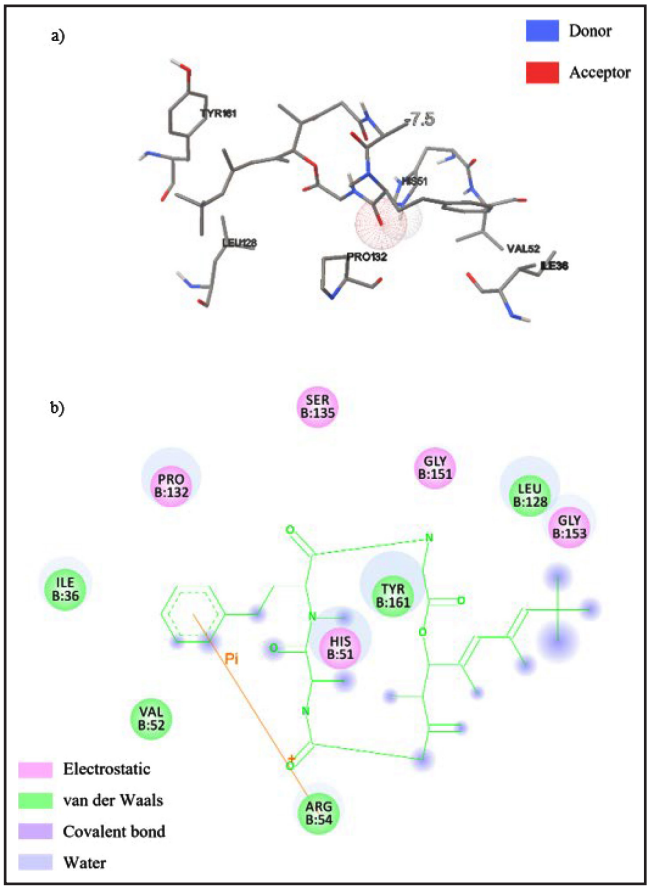

Figure 3. The interaction of antillatoxin B with DENV NS3/NS2B protein.

a) 3D scheme of the interaction of antillatoxin B with 2FOM, where a hydrogen bond can be found between antillatoxin B and the 2FOM residue HIS51. b) 2D scheme of the interaction of antillatoxin B with 2FOM, including pi-cation interaction and close contact.

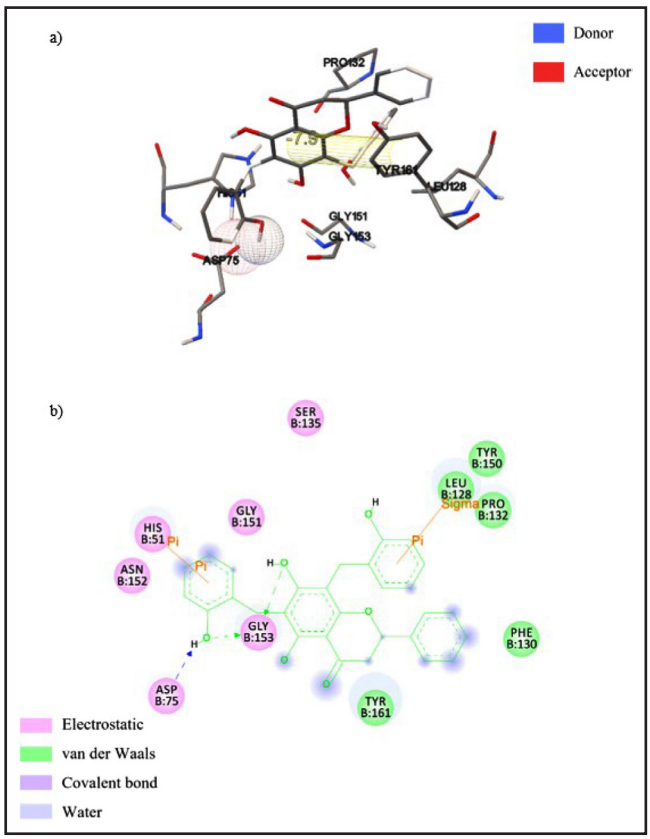

Figure 4. The interaction of dichamanetin with DENV NS3/NS2B proteins.
a) 3D scheme of the interaction of dichamanetin with 2FOM, where there is a hydrogen bond between dichamanetin and the 2FOM residue ASP75 and a pi-pi interaction with residue TYR161. b) 2D scheme of the interaction of dichamanetin with 2FOM, including hydrogen bonding, pi-sigma interaction, pi-pi interaction and close contact.

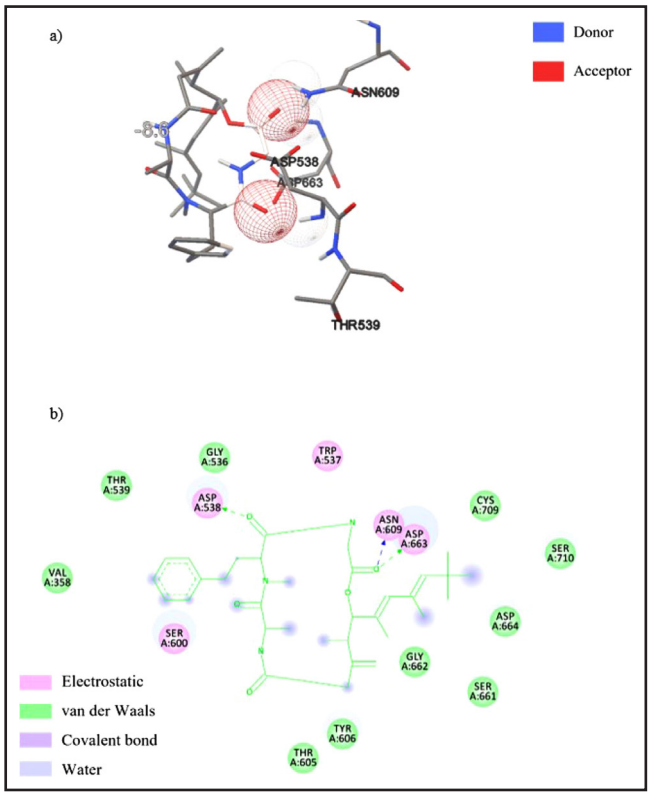

Figure 5. The interaction of antillatoxin B with DENV NS5 protein.
a) 3D scheme of the interaction of antillatoxin B with 2J7U, where hydrogen bonds can be found between antillatoxin B at residues ASP538 and ASP663. b) 2D scheme of the interaction of antillatoxin B with 2J7U, including hydrogen bonding and close contact.

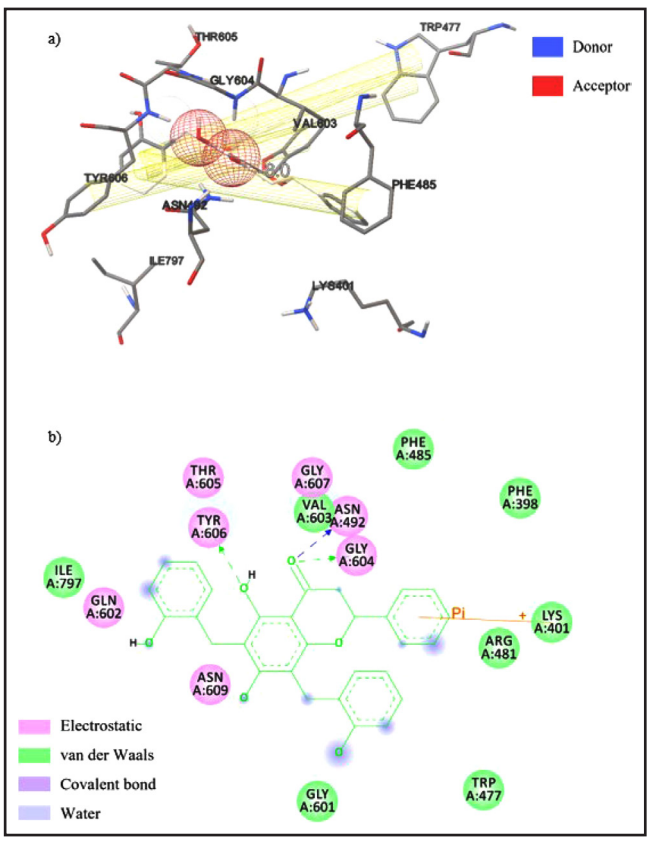

Figure 6. The interaction of dichamanetin with DENV NS5 protein.
a) 3D scheme of the interaction of dichamanetin with 2J7U, where there is a hydrogen bond between dichamanetin and the 2FOM residue GLY604 and pi-pi interactions at residues TYR606, ASN492, VAL603 and TRP477. b) 2D scheme of the interaction of dichamanetin with 2J7U, including hydrogen bonding, pi-cation interaction and close contact.

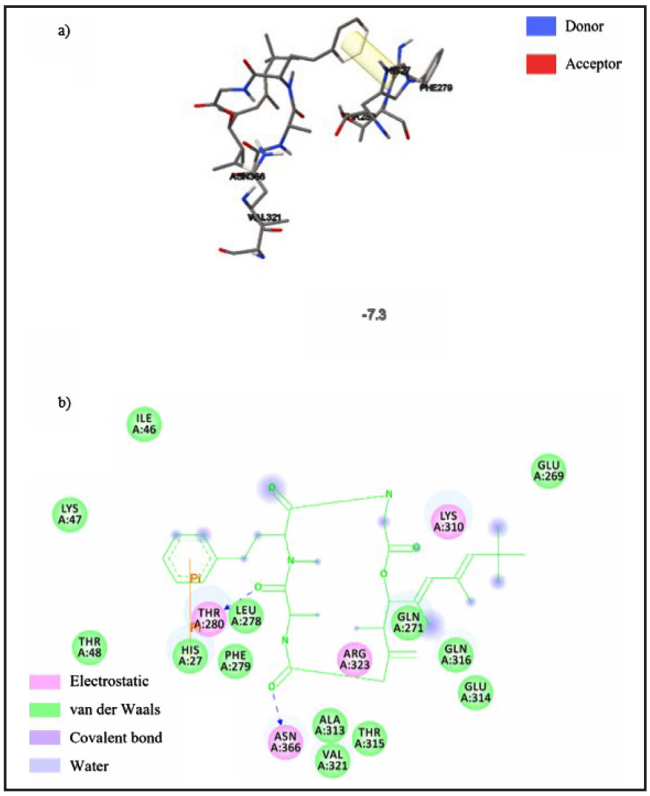

Figure 7. The interaction of antillatoxin B with DENV E protein.
a) 3D scheme of the interaction of antillatoxin B with E protein, with a pi-pi interaction between antillatoxin B and the E protein residue HIS27. b) 2D scheme of the interaction of antillatoxin B with E (1OKE) protein, including hydrogen bonding, pi-pi interaction and close contact.

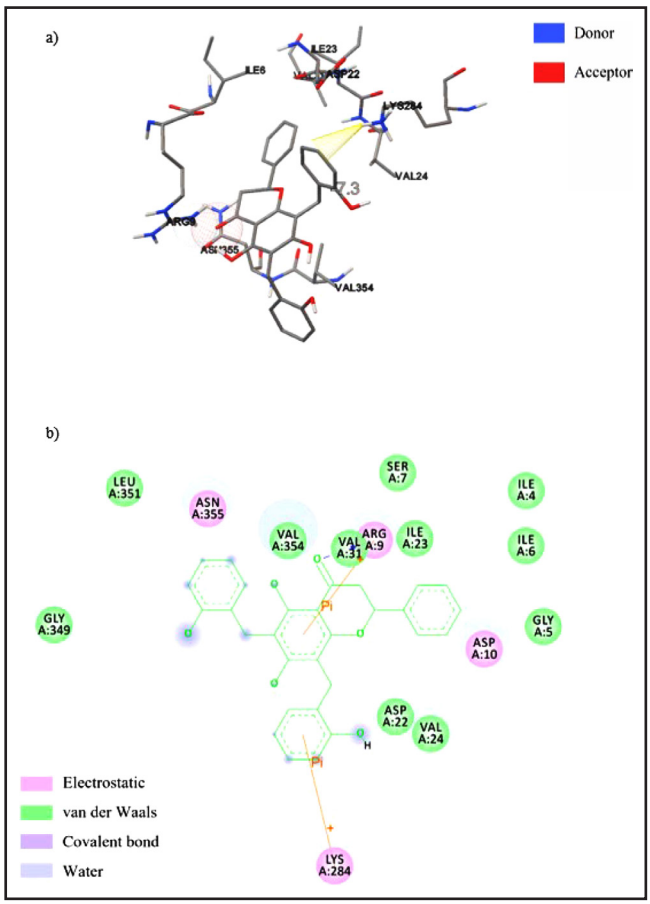

Figure 8. The interaction of dichamanetin and DENV E protein.
a) 3D scheme of the interaction of dichamanetin with E protein, with hydrogen bonding between dichamanetin and the E protein residue ARG9 and a pi-cation interaction at residue LYS284. b) 2D scheme of the interaction of dichamanetin with E (1OKE) protein, including hydrogen bonding, pi-cation interaction and close contact.

DISCUSSION

Earlier research in recent decades has identified a limited number of anti-arboviral agents exhibiting both mosquitocidal and anti-arboviral properties within mosquito cells. For instance, ivermectin, a broad-spectrum anti-parasitic drug, was discovered to have toxic effects on C6/36 cells and insecticidal activity on *Aedes* and *Anopheles* mosquitoes (Chaccour *et al*., 2010; Deus *et al*., 2012; Dong *et al*., 2019). In mammalian cells, ivermectin significantly reduced the infection of DENV-2, JEV, YFV, and CHIKV (Mastrangelo *et al*., 2012; Varghese *et al*., 2016); however, it showed no effect on ZIKV in mosquito midguts or cells when the concentration used for the assay was lower than the toxic level for cells or adult females (Dong *et al*., 2019). Cyclosporin, a broad-spectrum anti-arboviral (effective against DENV1-2, YFV, VSV, and ZIKA) (Qing *et al*., 2009; Barrows *et al*., 2016), did not inhibit ZIKV in C6/36 cells but displayed potent larvicidal activity against *Culex pipiens* (Weiser & Matha, 1988), adulticidal activity against *Ae. aegypti*, and toxicity in C6/36 cells (Dong *et al*., 2019). In the present study, the mosquito cell lineage C6/36 cells were used to test the EA extract of *Streptomyces* sp. KSF103 for potential anti-DENV activity. In the previous *in vitro* tests of mosquito cells and *in vivo* tests on different species of mosquitoes (Amelia-Yap *et al*., 2023), the EA extract demonstrated promising mosquitocidal activity, suggesting that this bacterial extract could be investigated further as an arboviral transmission-blocking agent.

Most studies examining mosquito immune responses to viral infections have used C6/36 cells (Sanchez-Vargas *et al*., 2004; Chu *et al*., 2006; Talarico & Damonte, 2007). The cell line has primarily been used for studies on insect-virus connections (Sanchez-Vargas *et al*., 2004), such as arbovirus detection, propagation, and analysis. One of the key characteristics of C6/36 cells is their ability to grow a diverse variety of mosquito-transmitted viruses and produce higher virus titers than any other cell (Brackney *et al*., 2010). Zika, dengue, and chikungunya are a few viruses studied using C6/36 cells. Virus propagation in the C6/36 cells directs the rational development of vaccines and therapeutics. In addition, C6/36 cell was used in this study because it can survive through the antioxidant defence and antiapoptotic effects after DENV infection for extended periods (Santana-Román *et al*., 2021).

Bafilomycin, produced from *Streptomyces griseus*, inhibits vATPases and has been proven to reduce DENV infection in various mammalian and insect cell lines (Kang *et al*., 2014) Bafilomycin inhibits Sindbis virus infection in mammalian BHK cells and lowers DENV by 80% in C6/36 cells (Mosso *et al*., 2008), though not in *Ae. albopictus* C7-10 cells (Hunt *et al*., 2011). Only in mammalian cells does the natural compound of *Streptomyces nanchangensis*, nanchangmycin, limits the production of numerous viruses in the Flaviviridae (DENV-2, ZIKV, and WNV) and Togaviridae (SINV, CHIKV) families (Rausch *et al*., 2017).

The potential of EA extract from *Streptomyces* sp. KSF103 to inhibit DENV-2 replication in C6/36 cells was assessed by pre-treating cells with varying doses 24 hours before infection. qRT-PCR results showed significant reductions in DENV-2 RNA levels, indicating the extract's ability to block viral entry. At its highest non-toxic concentration (14 µg/mL), the extract reduced infectivity by 95%. Increasing extract concentrations led to greater inhibition of viral replication, suggesting involvement of extracellular mechanisms. The extract may disrupt interactions between viral envelope glycoproteins and host cell receptors, impeding membrane fusion and viral entry (De La Guardia & Lleonart, 2014).

Treatment of C6/36 cells with *Streptomyces* sp. KSF103 EA extract after DENV-2 infection effectively suppressed viral RNA production, indicating post-infection antiviral activity. This may result from interference with the maturation and formation of infectious particles, preventing viral attachment to host receptors (Oo *et al*., 2019). Both pre- and post-treatment assays showed similar reductions in DENV-2 titers, with 75% and 76% inhibition at 3.5 µg/mL, respectively. At 0.875 µg/mL, post-treatment achieved 50% inhibition compared to 38% in pre-treatment. The maximum inhibition was below 90%, likely due to insufficient concentrations of active anti-DENV compounds (Kudi & Myint, 1999). Similar post-infection antiviral effects have been reported in previous studies (Muhamad *et al*., 2010; Low *et al*., 2011; de Souza Cardoso *et al*., 2013). These findings suggest the extract contains active component(s) with anti-DENV potential, which warrant further purification for development into a more potent arboviral transmission-blocking agent.

Zulkifli *et al*. (2023) and the present study investigated the antiviral activity of *Streptomyces* sp. KSF 103 extracts against DENV-2, using different extraction solvents and cell lines. Zulkifli *et al*. (2023) utilized a methanol extract tested on Vero cells for antiviral and cytotoxicity assays, with C6/36 cells used for virus propagation. The extract exhibited its strongest effect during the virus entry stage, with moderate activity at other stages of the viral replication cycle. In contrast, the present study used an ethyl acetate extract on C6/36 cells and observed concentration-dependent inhibitory effects in both pre- and post-treatment assays at comparatively lower concentrations. Although both extracts showed antiviral properties, the differences in solvent polarity, target cell types, and assay approaches likely contributed to the variation in observed efficacy.

Computational simulation suggested that antillatoxin B and dichamanetin exhibited the highest tendency to bind with DENV proteins. Antillatoxin B, a lipopeptide, has previously been reported as a neurotoxin (Li *et al*., 2001). Despite the absence of a study on its inhibitory property on viruses, lipopeptides have successfully inhibited human parainfluenza virus type 3 (HPIV-3), measles virus, influenza virus, and Nipah virus infection (Mathieu *et al*., 2017, 2018; Figueira *et al*., 2018a, 2018b). Recently, a lipopeptide has been proposed as a candidate antiviral for pre-exposure and early post-exposure prophylaxis for SARS-CoV-2 (de Vries *et al*., 2021). As a flavonoid, dichamanetin is well-known for its anticancer and antibacterial properties. It also exhibits notable antioxidant and anti-inflammatory effects, which are particularly relevant to its use as an anticancer agent as it modulates reactive oxygen species (ROS) and the NF-κB pathway to induce cancer cell death (Yong *et al*., 2013). While there is limited research on dichamanetin's direct antiviral effects, flavonoids as a class are widely recognized as effective antivirals, including against the dengue virus (DENV).

The pre-treatment assay showed that the EA extract of *Streptomyces* sp. KSF103 inhibited DENV-2 binding to cell receptors, likely through interactions with the E protein. Post-treatment results also indicated suppression of viral replication, possibly via interactions with NS2B/NS3 protease and NS5 proteins. These findings support the hypothesis that antillatoxin B and dichamanetin interact with both structural (E) and non-structural (NS2B/NS3, NS5) DENV proteins. As such, further molecular docking studies on their mechanisms of action may strengthen their potential as anti-DENV agents.

DENV proteases cleave at multiple sites, including NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5, as well as near signal sequences and junctions like C-prM and NS4A/NS4B (Mukhametov *et al*., 2014). The NS3 protease, key to replication and polyprotein processing, is a serine protease with a catalytic triad: HIS51, ASP75, and SER135 (Yildiz *et al*., 2013). Its N-terminal mediates protease activity, while the C-terminal supports NTPase and helicase functions (Oliveira *et al*., 2014). NS3 activation depends on the NS2B cofactor (Bollati *et al*., 2010), making the NS2B/NS3 complex a more relevant drug target than NS3 alone. However, NS2B's exact activation mechanism remains unclear (Li *et al*., 2005). Docking studies showed antillatoxin B and dichamanetin strongly bind the NS2B/NS3 complex, with energies of –7.5 and –7.9/ kcal/mol, respectively. Antillatoxin B forms H-bonds with HIS51 and GLY153, and pi–pi interaction with TYR161. Dichamanetin interacts via H-bonds with ARG55 and GLY153 and a pi–pi interaction with HIS51, a key active-site residue, supporting their potential inhibitory roles.

The DENV positive-strand RNA genome is replicated by the NS5 protein, which is known to be an RNA-dependent RNA polymerase (RdRp), in an asymmetric and semiconservative manner in which the anti-genome is only present in a double-stranded RNA replication intermediate (Mukhametov *et al*., 2014). It has been demonstrated through experiments that NS5 aids in the activities of NS3's RNA 50-triphosphatase (50-RTPase) and NTPase, and NS3 also triggers guanylyltransferase (Gtase) activity in NS5 (Khromykh *et al*., 2001; Issur *et al*., 2009). By activating some crucial enzymes, NS5 plays a crucial role in DENV replication, making it a promising option for developing antiviral drugs. The findings show interactions and close contact between NS5 and both compounds isolated from *Streptomyces* sp. KSF103 EA extract. In contrast to dichamanetin, which has a binding affinity equal to -8.4 kcal/mol, antillatoxin B displays slightly better interaction and has a higher binding affinity of -8.6 kcal/mol. From the result, antillatoxin B and dichamanetin have also been shown to interact with NS5 through pi-pi interactions, which strengthen their affinities for the protein. This outcome supports the previous findings on the intracellular anti-DENV activity of *Streptomyces* sp. KSF103 EA extract against DENV-2 after the internalisation of the virus into C6/36 cells. However, further investigation is required to reveal the precise biological mechanism of action on the *in vitro* replication of DENV due to treatment with the EA extract.

Domain III of the DENV E protein is a key target for blocking viral entry by preventing receptor binding (Crill & Roehrig, 2001). Molecular docking results showed that both antillatoxin B and dichamanetin interact with this domain, supporting a potential mechanism by which the *Streptomyces* sp. KSF103 EA extract inhibits viral internalisation. Dichamanetin demonstrated strong binding affinity (-7.3 kcal/mol) with domains II and III of the E protein, forming both pi–pi and pi–cation interactions. Antillatoxin B also formed stabilising pi–pi interactions with the E protein. These findings align with previous observations that the EA extract effectively inhibited DENV-2 attachment to C6/36 cells, suggesting its potential to block early stages of viral entry in mosquito cells.

Given that antillatoxin B and dichamanetin demonstrated inhibitory activity against the DENV-2 replication cycle, their anti-DENV potential could be extended to both in vitro applications and, potentially field trials. Chemical modifications may enhance their bioavailability, further improving their antiviral efficacy. Moreover, the low toxicity profile of the EA extract toward non-target organisms suggests that higher concentrations of these compounds could be safely employed to achieve effective DENV inhibition, potentially disrupting mosquito-to-human transmission. In summary, the findings support the potential of antillatoxin B and dichamanetin as promising lead compounds for the development of novel insecticidal agents targeting DENV infections.

Declaration of competing interest

The authors declare no conflict of interest.

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