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COMPUTATIONAL STUDIES OF POTENTIAL EBOLA VP40 INHIBITORS USING BIOACTIVE COMPOUNDS FROM MEDICINAL PLANTS OF MALAYSIA

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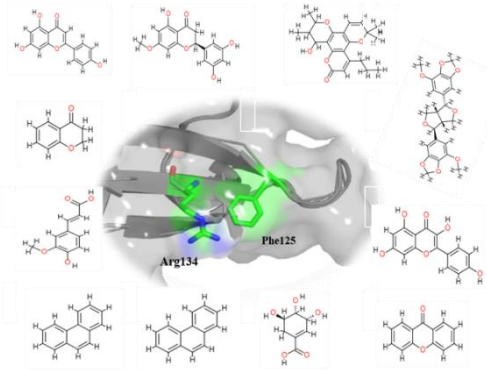
Graphical abstract



Abstract

Ebola virus (EBOV) belongs to Filoviridae family, a deadly virus that can cause severe viral haemorrhagic fevers (VHF) with a high fatality rate between 25 to 90 percent. Amongst EBOV proteins, the EBOV matrix protein VP40 is crucial in facilitating the transcription of the viral gene in the early stage of infection. To date, there is no cure for EBOV and available chemical drugs were known to cause severe side effects. It is known that bioactive compounds from natural products can potentially combat this viral disease with fewer side effects. Therefore, this study aims to screen 15 bioactive compounds of medicinal plants from Malaysia. These compounds were docked against the RNA active site (Phe125 and Arg134) on VP40 matrix protein using AutoDock Vina. The ADMET properties and the toxicity class of the compounds were predicted computationally, and the compounds with good oral bioavailability were chosen for docking simulations. The top three docked compounds namely apigenin, epiexcelsin and kaempferol have a binding affinity of -4.6, -4.4 and -4.3 kJ/mol respectively. Our MD simulation study showed that epiexcelsin is the best candidate among the three selected compounds. Binding free energy calculation via molecular-mechanics Poisson Boltzmann surface area (MM-PBSA) method showed that epiexcelsin has the lowest binding free energy of -56.503 kJ/mol compared to apigenin (-40.344 kJ/mol) and kaemferol (-27.329 kJ/mol). Our results suggest that epiexcelsin from local herbal plants can potentially be explored as a good candidate for further development of EBOV inhibitor targeting VP40.

Keywords: EBOV, Bioactive compounds, Apigenin, Epiexcelsin, Kaempferol, Molecular Dynamic Simulation, MM-PBSA

Abstrak

Virus Ebola adalah daripada keluarga Filoviridae yang boleh menyebabkan kadar kematian yang tinggi antara 25 sehingga 90 peratus disebabkan oleh jangkitan demam berdarah (VHF). Di antara protein-protein EBOV, interaksi protein VP40 adalah penting untuk proses transkripsi gen viral pada peringkat awal jangkitan. Sehingga kini, tiada penawar yang khusus untuk mengatasi penyakit ini dan ubat-ubatan yang sudah

dipasarkan telah diketahui mempunya kesan sampingan yang berbahaya. Diketahui bahawa sebatian bioaktif daripada sumber asli adalah berpotensi untuk menghalang penyakit ini dengan kesan sampingan yang lebih rendah. Oleh itu, kajian ini bertujuan untuk menyiasat 15 sebatian bioaktif daripada pokok-pokok herba dari Malaysia. Sebatian ini telah menjalani simulasi berkomputer terhadap residu tapak aktif RNA (Phe125 dan Arg134) pada protein VP40 menggunakan AutoDock Vina. Kriteria ADMET dan golongan kelas toksik telah diramalkan dan sebatian yang dipilih telah mempunyai serapan bio-ketersediaan yang baik untuk simulasi dok. Tiga dok sebatian (apigenin, epiexcelsin dan kaempferol) mempunyai ikatan tenaga terendah iaitu -4.6, -4.4 dan -4.3 kJ/mol. Simulasi dinamik molecular menunjukkan bahawa epiexcelsin adalah antara calon yang paling sesuai antara tiga sebatian yang dipilih. Pengiraan ikatan tenaga bebas sebatian kompleks tersebut menggunakan kaedah Poisson Boltzman surface area (MM-PBSA) menunjukkan epiexcelsin mempunyai tenaga bebas sebanyak -56.503 kJ/mol berbanding apigenin (-40.344 kJ/mol) dan kaemferol(-27.329 kJ/mol). Penemuan kami telah mencadangkan bahawa epiexcelsin daripada pokok herba tempatan mempunyai potensi untuk diexplorasi sebagai calon sebatian dalam pembangunanubat-ubatan baru yang mensasarkan protein VP40 sebagai penentang virus Ebola.

1.0 INTRODUCTION

EBOV disease has been a universal public health treat since its discovery. The World Health Organization (WHO) has affirmed that the EBOV outbreal as a "Public Health Emergency of International Concern" on 8th August 2014 [1]. The research and development of vaccines and therapeutics for the Ebola Virus (EBOV) has been going on for decades [1] to combat EBOV disease. To date, there is no precise antiviral management or vaccination for the disease [2]. EBOV has been recognized worldwide as one of the most dangerous viral diseases due to its non-specific symptom, high mortality rate and severe morbidity [2]. Any country including Malaysia has the likelihood of a probable EBOV outbreak. The Ministry of Health (MOH) has taken steps to strengthened and enhanced the preparedness and response to mitigate the outbreak. Ebola is listed as one of the mandatory notifiable disease in Malaysia under the Prevention and Control of Infectious Disease Act 1998 (Act 342) [3].

EBOV are single-stranded, lipid-enveloped, non-segmented and negative-sense RNA virus which belongs to Filoviridae family. The length of EBOV can vary from 80 nm and up to 14,000 nm. Five species of EBOV has been identified namely Sudan, Bundibugyo, Zaire, Ivory Coast and Reston [6]. Infection of EBOV will disable the immune system first and subsequently causes severe viral haemorrhagic fevers (VHFs), focal necrosis of the liver, kidney and spleen, sudden shock and bleeding diathesis in human and non-human primates [7, 8]. It can enter the human body via the skin, mucus membranes and are also highly contagious, EBOV disease spreads easily through bodily fluids such as saliva, blood, breast milk, stool and semen [9].

EBOV genome codes for seven structural proteins that can potentially become drug targets namely the nucleoprotein (NP), viral protein (VP) 35, matrix protein VP40, glycoprotein (GP), VP30, VP24, and polymerase protein (L) [10]. These seven genes encoded in their negative-sense RNA genome will be utilized for replication when these pleomorphic filamentous viruses enter the host cell through its membrane-embedded glycoprotein. Among these proteins, VP40 is the most expressed EBOV protein that can mediate particle formation, regulates the viral budding as well as important in virus structure and stability [8]. It is located under a viral bilayer between the envelope and nucleocapsid in the matrix space [8]. It is also required for viral assembly and viral egress. In host cells, VP40 can interact with its RNA metabolism during the replication process. With this interaction, the formation of octamer and replication of EBOV can be promoted [10]

Two important residues of VP40 protein namely Phe125 and Arg134 have been reported as key residues in mediating the VP40-RNA interaction. These two residues are positioned side by side in the folded protein as depicted in Figure 1 [12]. This RNA binding of VP40 also follows the induced fit pathway as mutation on these residues was found to affect the RNA-binding process [13]. Furthermore, this VP40-RNA binding is an important process to facilitate the transcription of the viral gene in the early infection stage [14]. Thus, this RNA binding site is one of the promising targets in designing EBOV VP40 inhibitors.

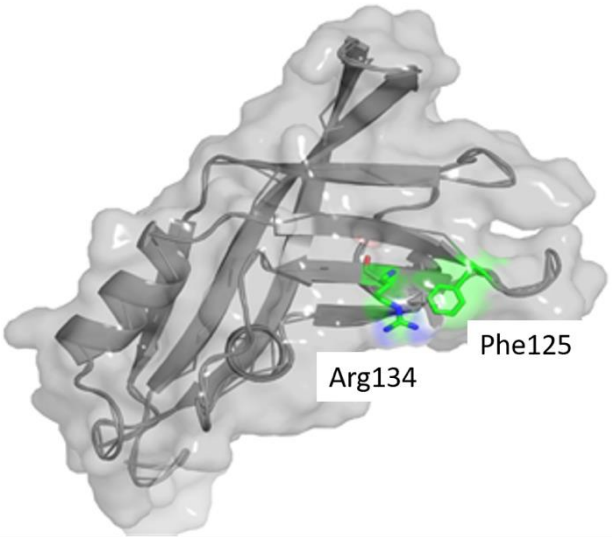


Figure 1 The matrix protein VP40 from Ebola Virus with PDB code: 1H2C and the important binding residues (Phe125 and Arg134)

Many synthetic drugs have been produced and tested against EBOV but none of it has proved to be highly effective against the virus especially in humans. For example, BioCryst has developed several experimental drugs including the synthetic adenosine analogue (BCX4430) to help inhibit EBOV [15]. Apart from that, there has been a utilisation of the strain from the tobacco plant using ZMapp which combined the individual monoclonal antibodies, MB-003 (Mapp) and ZMAb (Defyrus/PHAC) with a 43% successful rate however it has not been tested in humans [16]. Other several tested EBOV synthetic drugs on animals such as T-705 (favipiravir), FGI-106 and TKM-Ebola has been tested on mammalian cells and monkeys [17]. Although TKM has advanced into clinical trial phase I, it was suspended in phase II as it produced adverse effects. Drugs that were tested on humans was CMX001 and Lamivudine. CMX001 has been approved by the FDA and has cured one patient but unfortunately, the funding from the manufacturer has been halted whereas 13 out of 15 patients survived using Lamivudine only until the end of the course. Besides that, Ribavirin which has been used to treat several diseases showed poor results against EBOV [18].

In addition, synthetic drugs are prone to give negative effects on natural compounds from natural resources. Some of the side effects are the resistance development towards viral proteins, recurrent development and viral latency [19]. Therefore, exploiting bioactive compounds in medicinal plants may serve as an alternative to EBOV inhibitors. For the past 10 years, a lot of studies were concentrating on EBOV VP40 inhibitors research in the NCBI database. However, limited papers discussed the plant bioactive compounds from medicinal plants from Malaysia as inhibitors. This indicated that the search for new natural compounds as a cure to this disease is needed extensively because currently there is no specific treatment for this type of disease.

Thus, this study was set to explore bioactive compounds found in Malaysian medicinal plants as potential VP40 inhibitors. Molecular docking, molecular dynamic simulations and MM-PBSA approach were utilized to explore potential compounds that may exhibit good binding affinity towards VP40. Our results suggest that epiexcelsin is a promising compound for further analysis via experimental means.

2.0 METHODOLOGY

2.1 Target Protein EBOV VP40 Model

The crystal structure of matrix protein EBOV VP40 of resolution 1.6 Å was retrieved from Protein Data Bank (PDB) with PDB code: 1H2C. The structure was visualized using PyMOL to remove the heteromolecules from the protein structure [20]. The binding site residues (Phe125 and Arg134) were included in the docking grid due to their importance as stated in the previous studies [8, 21-29]. Extraneous water molecules were removed from the protein using AutoDockTools (ADT) software [30]. Hydrogen atoms were added and the partial charges (Kollman and Gasteigher charges) were computed for the protein system stabilization. The molecules were then exported and operated in PDBQT format.

2.2 Ligand Preparation

A total of 15 bioactive compounds commonly found such as flavonoids (flavones and flavonols), lignans and phytoestrogens were investigated in the current study. Among them are the herbal and vegetable plants mainly the leafy areas and some of them are known for their anti-fungal properties which also demonstrated good anti-viral activities [31] as summarized in Table 1.

Table 1 Fifteen bioactive compounds from Malaysian local plants (See Supplementary info for details)

No.	Bioactive Compounds	Pubchem CID
1	Apigenin	5280443
2	Blumeatin	70696494
3	Calanolide	1201
4	4-Chromanone	68110
5	Coumarin	323
6	Curcumin	969516
7	Epiexcelsin	489948
8	Ferulic acid	445858
9	Gallic acid	370
10	Gallocatechin	65084
11	Kaempferol	5260863
12	Phenanthrene	995
13	Protocatechuic acid	72
14	Shikimic acid	8742
15	Xanthone	7020

The 2D structures of the bioactive compounds were retrieved from PubChem Compound Database. The 2D SDF format ligand structures were converted to 3D structure in PDB format and prepared accordingly for the molecular docking step. The Kollman and Gasteigher charges were computed for all ligands. Torsion roots and the rotatable bonds of the ligand's torsion angles were assigned to reduce the difficulty and time constraint when ligands are being rotated.

2.3 Molecular Docking of Target Ligand to EBOV VP40

Fifteen chosen compounds were docked at the binding site Phe125 and Arg134 of the target protein molecule using AutoDock Vina version 1.1.2 [32]. In the initial step, ADT was used to assign polar hydrogen atom and Kollman charges to both protein receptor and ligand while non-polar hydrogens were merged with the structure. The binding site was specified in the grid map using AutoGrid in ADT at 1 Å as the default spacing. The grid box of point x, y and z dimensions wheels of 1.606 x 17.194 x 25.857 were enough to cover the specific binding site of the target molecule which was Phe125 and Arg134. The maximum number of binding modes was set at 10 while the default value was used during simulation. The complex structures were ranked according to the binding energy, from the lowest to the highest (lowest binding energy is preferable). The top three ligands having the lowest binding energy were selected for further analysis.

2.4 ADMET Analysis

The physicochemical and pharmacokinetic analysis were predicted for all ligands using ProTox-II website [33]. The website gives information such as the molecular weight, number of hydrogen donors and acceptors, toxicity class and toxicity testing for mutagenicity, carcinogenicity, immunogenicity and hepatogenicity. SwissADME was used to further clarify the pharmacokinetics and bioavailability of the compounds [34]. The compounds were also being assessed for Lipinski's drug-likeness rules of violation [39].

2.4 Molecular Dynamics Simulation

The resulted protein protein-ligand complex with good ADMET properties and lowest binding energy was selected for the subsequent MD simulation study. MD simulations were performed in GROMACS version 5.1.4 using the GROMOS96-54a7 force field [35-38]. The VP40-ligand complexes were simulated in cubic box and solvated using SPCE water model. Appropriate counterions were added to neutralize the system. The neutralized system underwent energy minimization to ensure the system was in appropriate geometry to avoid steric clashes during MD simulation. Then, the system was equilibrated at 300 K for 50 ps by using the Verlet cutoff scheme. Berendsen barostat was used for the equilibration with the isotropic pressure coupling type. The compressibility was set at 4.5 x 10^{-5} bar^{-1}.

The equilibrated system was subjected to molecular dynamics simulation for 20 ns at a constant temperature of 300 K. The simulation was run using Parinello-Rahman barostat, where the pressure coupling between protein and ligand groups was isotropic with the compressibility of 4.5 x 10^{-5} bar^{-1}. Verlet neighbour scheme was used while ring systems and stiff bonds were controlled using the LINCS algorithm. A time step of 2 fs was used to run the systems and was simulated in triplicates.

Results of the simulations, as well as the trajectory, were analysed using GROMACS tools, VMD and PyMol software. Some locally written scripts were used to analyse the simulation. In addition, the free binding energy analysis between the ligand and VP40 was analysed using the MM-PBSA approach through g_mmpbsa tools [37].

3.0 RESULTS AND DISCUSSION

3.1 Molecular Docking Analysis

Molecular docking analysis was performed to estimate the binding affinity of the ligands toward the target site of VP40. The docking scores of the 15 selected compounds are presented in Table 2 where the compounds are being ranked based on their docking scores. The results show that apigenin and blumeatin, both have the lowest binding energy of -4.6 kcal/mol. This is followed by epiexcelsin and kaempferol with docking scores of -4.4 and -4.3 kcal/mol respectively. Epiexcelsin has the closest interaction (1.8 Å) with Arg134 via one hydrogen bond. The observed interaction stipulated that only eight compounds (epiexcelsin, kaempferol, xanthone, coumarin, protocatechuic acid, gallic acid, 4-chromanone and shikimic acid) formed favourable interaction with Arg134 with a corresponding distance ranging from 1.8 to 2.2 Å.

Table 2 The molecular docking score analysis (lowest to highest) and the interacting residues

Bioactive Compounds	Binding energy (kcal/mol)	Binding Residues
Apigenin	-4.6	Thr123, Phe125*, Arg134*
Blumeatin	-4.6	Thr123, Phe125*, Arg134*
Epiexcelsin	-4.4	Thr123, His124, Phe125*, Gly126, Arg134*, Asn136
Kaempferol	-4.3	Thr123, Phe125*, Arg134*
Gallocatechin	-4.1	Thr123, Phe125*, Arg134*
Phenanthrene	-4.1	Phe125*, Arg134*
Xanthone	-3.9	Thr123, Phe125*, Arg134*
Calanolide	-3.8	Phe125*, Gly126, Arg134*, Ala156
Curcumin	-3.8	Phe125*, Arg134*
Coumarin	-3.7	Thr123, Phe125*, Arg134*
Protocatechuic acid	-3.5	Thr123, Phe125*, Arg134*

Bioactive Compounds	Binding energy (kcal/mol)	Binding Residues
Gallic acid	-3.5	Thr123, Phe125*, Arg134*
4-Chromanone	-3.4	Phe125*, Arg134*
Ferulic acid	-3.4	Phe125*, Arg134*
Shikimic acid	-3.4	Thr123, Phe125*, Arg134*

* the important residues in RNA binding activity.

3.2 ADMET Analysis

The ADMET analysis for the ligands is tabulated in Table 1 (in Supplementary data). All 15 ligands have good drug-likeness properties as they were following the Lipinski Rule of Five (MW <500, Hydrogen bond donors <5, Hydrogen bond acceptor <10, and LogP <5) and Verber Rule (<10 rotatable bonds) for its bioavailability [38-39]. All fifteen compounds are suggested to be highly absorbed in the gastrointestinal of humans and are soluble except phenanthrene. Apart from that, most of the ligands did not have PAINS alerts except for gallic acid, gallocatechin and protocatechuic acid. The other 12 compounds with zero PAIN alerts indicated that most of them do not have any unfavourable moieties or fragments in their chemical structure [40].

The toxicity of the compounds was further confirmed using ProTox-II web server in which the toxicity class can be well studied. The result shows that only four compounds (calanolide, coumarin, phenanthrene and xanthone) has LD_{50} of <1500 mg/kg but all compounds have toxicity class between 3 to 6 (1: Highly toxic, 6: Non-toxic) [33]. Only gallocatechin, shikimic acid, kaempferol and apigenin possessed no toxicity whereas other compounds had inactive toxicity (immunogenicity, mutagenicity, carcinogenicity and hepatogenicity).

3.3 Molecular Dynamics Simulation

Based on molecular docking and ADME analysis, three compounds namely apigenin, epiexcelsin and kaempferol that have good docking scores and overall bioavailability were selected for MD analysis. Blumeatin was not chosen even though it has good docking energy because it is more toxic as it showed immunotoxicity when compared to apigenin (Supplementary data, Table 1). Meanwhile, gallocatechin has unfavourable chemical structure moieties (with 1 PAINS alert), therefore only three compounds (apigenin, epiexcelsin and kaempferol) were chosen for MD simulation and analysis.

To investigate the conformational stability of the protein-ligand complex, MD simulation for each of the complex structures was monitored throughout triplicates of 20 ns simulation trajectories. The root-mean-square deviation (RMSD) analysis gives the overall picture of how much the 3D structure of VP40 fluctuates and changes its conformation throughout the simulation. The RMSD value showed that the interaction between VP40 and apigenin (black) is at ~5 and 6 Å at the end of the simulation whereas epiexcelsin (green) is at ~3.5 to 4.0 Å (Figure 2). However, kaempferol (red) only showed stabilization at ~4 to 4.5 Å but was relatively unstable toward the end of the simulation, as indicated by increasing RMSD values. All three compounds demonstrated convergence at 15 to 20 ns which indicates the stability point of those three simulated interactions. These RMSD results explained that the movement of those compounds are small and therefore showing the strength of those compounds within the binding site of VP40. Apart from that, it is studied to gauge the dynamic stabilities of the simulated system which in this case the binding stabilization can be seen in the last 5 ns.

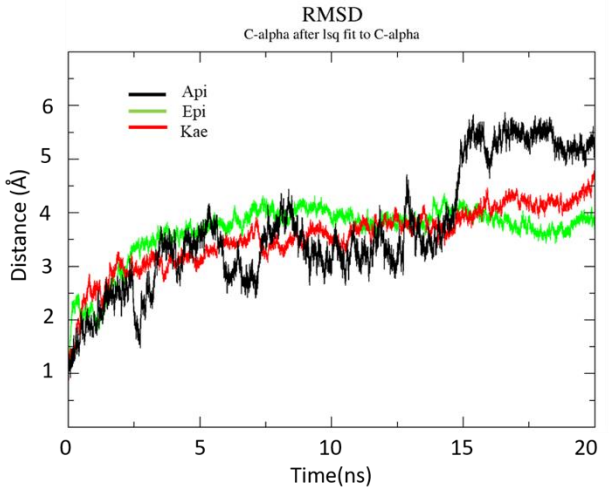


Figure 2 RMSD plots for three compounds throughout 20 ns simulation system. (Api: apigenin, Epi: epiexcesin, Kae: kaempferol)

RMSF analysis were carried out to clarify the structural fluctuations for each of the amino acid residues. Lower RMSF values (Figure 3) were demonstrated for epiexcelsin throughout the simulation and this is consistent with RMSD plots in which this compound exhibited small fluctuation and stable RMSD. It was observed that Pro85 and Lys104 strongly interacted with epiexcelsin with minor fluctuation below 3.5 Å, noticeably lower RMSF at these residues compared to apigenin and kaempferol. In addition, epiexcelsin also showed minor fluctuation throughout the simulation. However, apigenin demonstrated major fluctuation at residues Pro85 and Lys104. The compounds fluctuated in a similar pattern between residues Tyr120 to Pro140, ranging below 3 Å, suggesting that apigenin is more flexible and not strongly bound to the target site of VP40. The binding free energy between the compounds and VP40 was then calculated using MM-PBSA approach to determine the favourability of the interaction. The last 5 ns from the RMSD plots were extracted for free binding energy calculation.

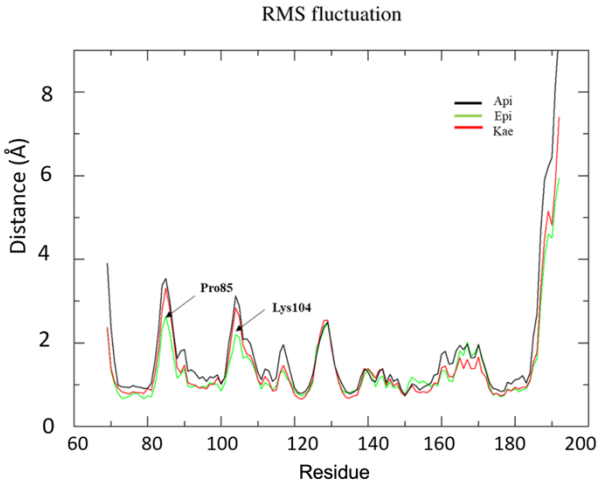


Figure 3 RMSF plots for three compounds throughout 20 ns simulation system

The role of RNA binding and formation of VP40 octamer can be illustrated by the plotted distance between VP40 important binding residues (Phe125 and Arg134) and the three selected compounds. Apigenin and epiexcelsin in Figure 4 were situated at a short distance from both residues with minimal variation. Only kaempferol was deviated further away at the early stage of the simulation and started associating with the protein at a closer, stable distance of 20 Å. At 15 ns timeframe, all three compounds reached minimum distance with each other where the compounds formed relatively stronger binding to the target protein compared to the initial configuration. It is obvious from the MD simulation that epiexcelsin is the most promising candidate as it displays close contact and stable interaction throughout the simulation. Thus, it may potentially have the ability to suppress the binding of RNA to VP40 [41].

The MD simulation trajectory also showed that the number of residues start to move closer at three subsequent time frames (19.29, 19.76 and 20.0 ns) as shown in Figure 5. Apart from Arg134 and Phe125, the interacting residues such as Lys86, Gln170 and Thr173 can be seen to move closer to apigenin by the end of the simulation (Figure 5 (A)). Similar results can be seen with epiexcelsin in which the distance between Gly126 and epiexcelsin has reduced when reaching 20 ns simulation (Figure 5 (B)). However, this was different for kaempferol (Figure 5 (C)) where Leu117 and Arg137 has the closest interaction at 19.76 ns and then Leu117 moved slightly farther away at 20 ns whereas Arg137 maintained its interacting distance.

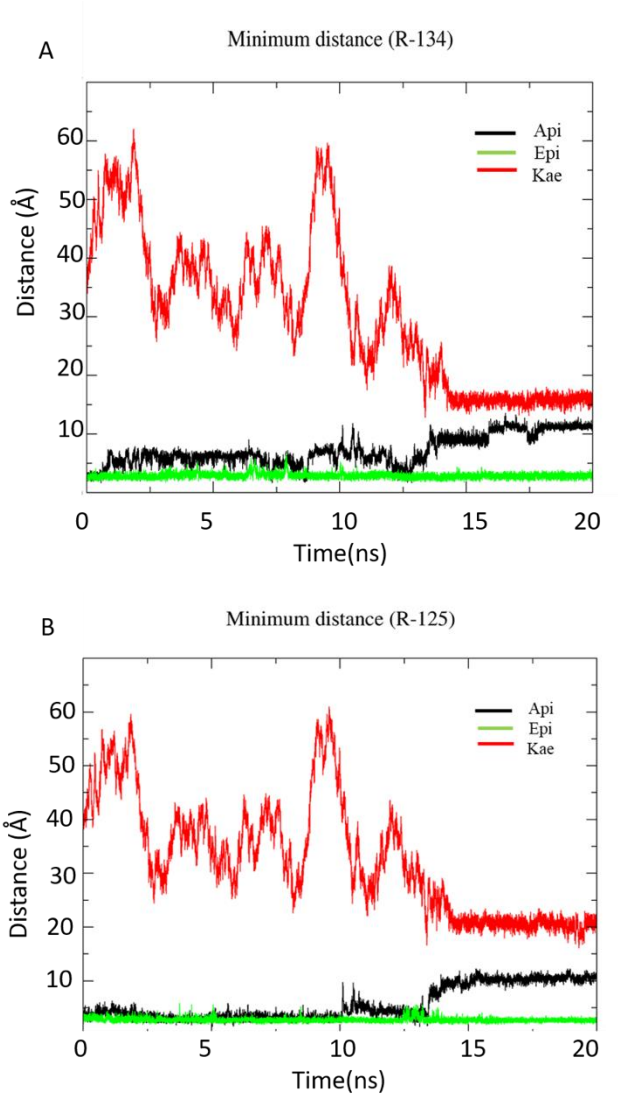


Figure 4 A) Distance between Arg134 of VP40 with selected compounds throughout the simulation. B) The distance between Phe125 of VP40 with selected compounds throughout the simulation

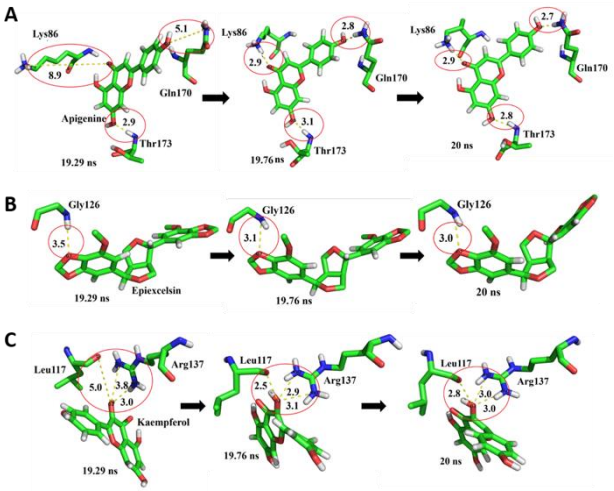


Figure 5 MD simulation and the hydrogen bond distance at different timeframe by the end of the simulation. (A) Apigenin, (B) Epiexcelsin and (C) Kaempferol

To further determine the favourability of the interaction, we calculated the binding free energy between the ligands and VP40 through MM-PBSA approach using the g_mmpbsa tool. From the results of MM-PBSA calculation, all three compounds gave lower negative binding free energy values between -27 to -56 kJ/mol which demonstrated favourable binding between the ligands and VP40 (Table 4). Epiexcelsin has the lowest RMSD values at the end of the simulation and this is further being confirmed by having the lowest free binding energy which is -56.503 kJ/mol compared to apigenin and kaempferol. Overall, the three systems have stable RMSD fluctuation and further validation by MM-PBSA calculation indicates that the binding of the ligands to VP40 at the RNA binding site maintained the stability of the protein.

Table 4 Average binding free energy for each ligand in the last 5 ns of the simulation

Compounds	Average binding free energy (kJ/mol)
Apigenin	-40.344
Epiexcelsin	-56.503
Kaempferol	-27.329

4.0 CONCLUSION

In this study, in silico methods have been used to investigate bioactive compounds derived from plants against EBOV VP40 RNA binding sites using molecular docking and molecular dynamics simulation approach. Our result revealed that epiexcelsin exhibited the lowest docking energy of -4.4 kJ/mol compared to apigenin and kaempferol. Epiexcelsin remained favourably associated with Phe125 and Arg134 throughout the simulation. Our MM-PBSA results also showed that epiexcelsin has the lowest free binding energy of -56.503 kJ/mol. In conclusion, we suggest this compound as potential lead that could be utilized as effective VP40 inhibitors to combat EBOV disease.

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