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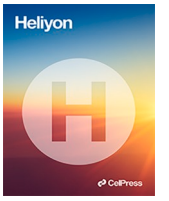

Alpha-mangostin, piperine and beta-sitosterol as hepatitis C antivirus (HCV): In silico and in vitro studies

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ABSTRACT

Hepatitis C is still a serious liver case of health. Up to now the development of anti-Hepatitis C Virus (HCV) drugs is challenging, especially the development of natural material compounds as anti-HCV. In the present study, we evaluated the probability of α-mangostin, piperine, and β-sitosterol as anti-HCV with the in silico and in vitro approaches. Molecular docking was performed between nonstructural protein 5B (NS5B, PDB ID 3FQL) with α-mangostin, piperine, and β-sitosterol by Autodock Tools® and BIOVIA Discovery Studio®. Subsequently, molecular dynamics simulations were conducted for 200 ns, evaluating the dynamic interaction between the ligands and the viral protein NS5B. Furthermore, compound characterization at the hepatocarcinoma cell line was employed. α-Mangostin with NS5B complex demonstrated the most negative binding free energy value based on MM-PBSA calculation with a value of −9.13 kcal/mol. In vitro test showed that IC_{50} of α -mangostin was 2.70 ± 0.92 μM, IC_{50} of piperine was 52.18 ± 3.21 μM, IC_{50} of β-sitosterol was >100 μM. α-Mangostin can serve as a valuable lead compound for further development of the anti-HCV.

1. Introduction

Hepatitis C is a serious liver malady, caused by the flaviviridae family RNA virus [1,2]. This virus has infected over than 169 million humans in the globe [3]. More than 50% patient who are infected undergo a chronic stage and potentially develop into a cirrhosis and fibrosis of liver [4]. Up to now, there is no available vaccine for preventing and treating HCV infection. The available treatment for HCV diseases, such as simeprevir, sofosbuvir, and ribavirin, mostly targeting to inhibit the HCV replication [5–8]. The current HCV therapy are relatively expensive with the various side effects upon the prolonged medication use. On the other hand, HCV easily

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develops resistance, making the current treatment's effectiveness futile. Thus, there is a need for alternative development of HCV drugs that are affordable with better safety and higher efficacy [9–11].

Currently, the use of medicinal plants as the antiviral is intensively developed, in which the active substances (phytochemicals) in them are able to inhibit viral replication and thus increase the body's immunity against viruses that enter the body [6]. Mangosteen (*Garcinia mangostana Linn.*), a plant widely found in Southeast Asia countries such as Indonesia, has been traditionally used for diarrhea, chronic ulcer, skin infection treatments, and wounds. The main phytochemical in mangosteen is α-mangostin [12–14]. α-Mangostin is shown to have antioxidant, anticancer and antiviral effect [15,16].

β-Sitosterol (phytosterol in plants) has been developed into a nutraceutical supplement. It has been shown to have anti-inflammatory, antimicrobial, immunomodulatory and antidiabetic effects [17]. Recent findings, showed the effect of β sitosterol to actively inhibit the white spot syndrome virus [17–19].

Piperine, an alkaloid in the Piperaceae family such as black pepper (*Piper nigrum*) or white pepper (*Piper alba*), has been shown to have anti-inflammatory, antimicrobial, antiulcer, anticancer and anti-infective activities [20,21]. In addition, it exhibits an antiviral activity against Ebola and dengue viruses, making it a potential candidate for further development as an antiviral agent [22].

In the present research, the molecular mechanism of α-mangostin, β-sitosterol and piperine as anti-hepatitis C were evaluated by in silico and additionally in vitro approaches.

2. Materials and methods

2.1. In silico experiments

2.1.1. Preparation of ligands

The ligands used in this study (α-mangostin, β-sitosterol and, piperine and sofosbuvir (commercial anti-HCV drug)) were obtained from www.molview.org in MDL format. The structures were optimized and energy minimized by using Gaussian® and Avogadro® software.

2.1.2. Preparations of protein target

The hepatitis C protein used in this study was NS5B protein (PDB ID: 3FQL) of HCV. Hydrogen was added, and the water molecule was removed from the molecule prior to the docking experiment [23].

2.1.3. The validation of the method (molecular docking)

This process was done by re-docking the native ligand to the protein target using AutoDock® 4.2 software. In this process, the RMSD value was limited below 2 Å.

2.1.4. Molecular docking

Molecular docking between ligands and protein targets was performed by using MGLTools® 1.5.6 equipped with AutoDock® 4.2 software. It was done using parameters that had been validated against the protein target by searching for 100 random conformations then the best conformation was visualized and analyzed by using BIOVIA Discovery Studio® 2021.

2.1.5. Molecular dynamics simulation

The best conformation of the ligand-protein complex on molecular docking was further characterized with molecular dynamics simulation using Gromacs® 2021.3 software. Ligand parameterization was done by using AnteChamber Python Parser interfacE® (ACPYPE®). Addition of water as solvent as well as sodium and chloride ions to mimic intracellular processes performed prior to the 200 ns dynamic simulation.

2.1.6. ADMET prediction of compounds

This process was performed by ADMET predictor (pkCSM online at https://biosig.lab.uq.edu.au/pkcsm/prediction). This application could predict the ADMET of the compound by using input in the SMILES format. The pharmacokinetic properties were further evaluated. Then, the toxicity of the compounds was also predicted, such as toxicity for humans, bacteria, rats, and minnows.

2.2. In vitro anti-hepatitis C virus activity

2.2.1. Compounds preparation

The compounds used in this research were the α-mangostin reference compound (Markherb®, Bandung, Indonesia), β-sitosterol reference compound (Markherb®, Bandung, Indonesia), and piperine reference compound (Markherb®, Bandung, Indonesia) and sofosbuvir (Sigma Aldrich®) as the positive control.

2.2.2. Cell culture and hepatitis C virus -propagation

Hepatocarcinoma cells Huh7it were grown in DMEM (GIBCO Invitrogen®), supplemented with 150 μg/mL Kanamycin (Sigma Aldrich®), 10% of Fetal Bovine Serum (Biowest®) and non-essential amino acids (GIBCO Invitrogen®) and maintained in 5% CO_2 at 37 °C. Hepatitis C virus JFH1a (Genotype 2a strain) was infected into the cell culture. After the third and fifth days of the incubation, the supernatant was collected concentrated, and the titers of HCV was determined for anti-hepatitis C virus assay [24,25].

2.2.3. Anti-hepatitis C virus assay

Hepatocarcinoma cells Huh7it were seeded (5.4×10^4 cells/well) and further incubated for 24 h. HCV with a titer of 6.9×10^6 (MOI 0.1) was added to the cells. The mixture of HCV and samples was then incubated for 2 h. Then, the medium of the cells was renewed. Subsequently, the test compounds with various concentrations were added to them and further incubated for 48 h.

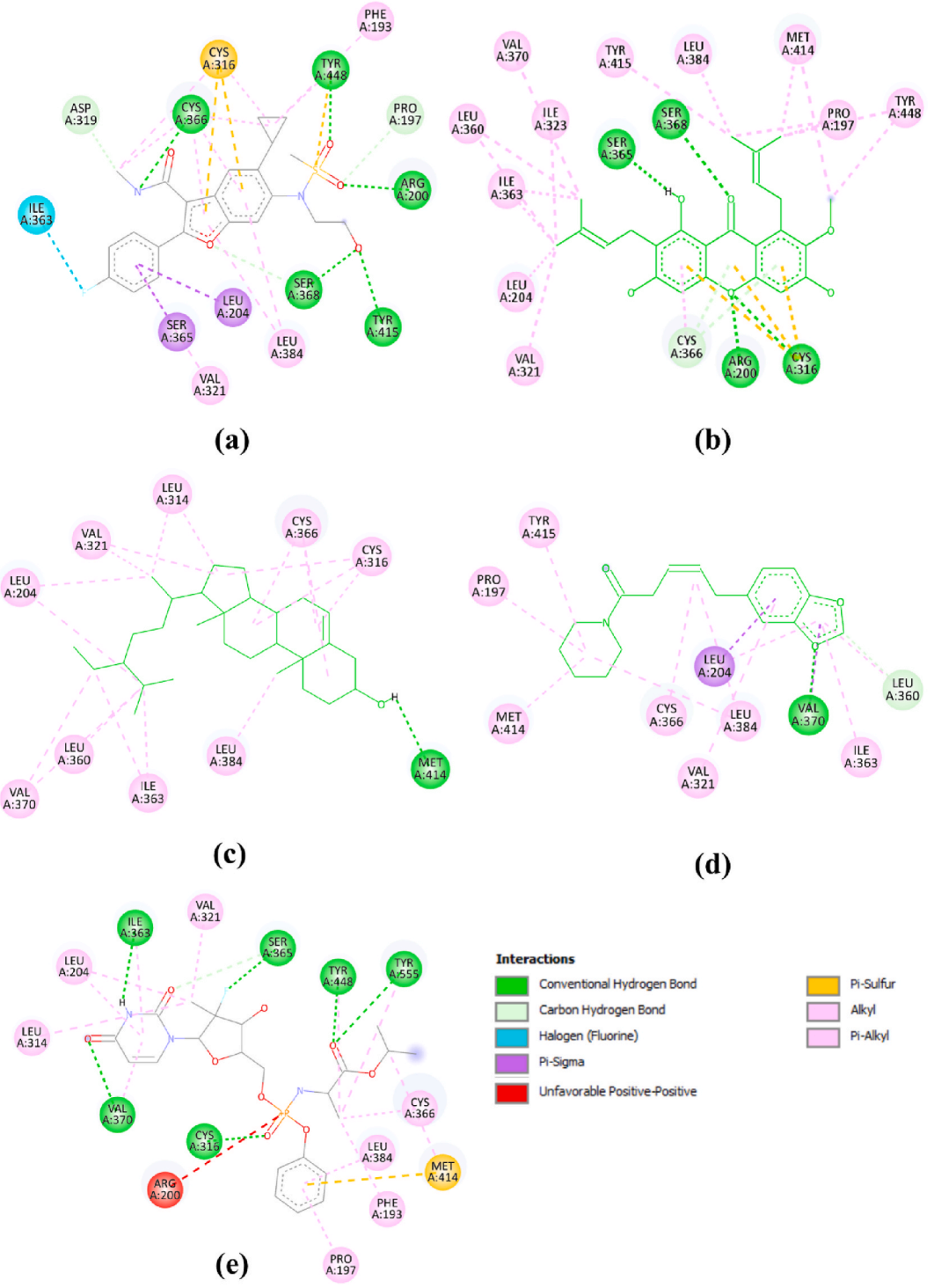

Fig. 1. Visualization of 2D (two-dimensional) of molecular docking interaction of ligands with NS5B protein (PDB ID 3FQL) (a) Native ligand (HCV-796), (b) α-mangostin, (c) β-sitosterol, (d) Piperine, (e) Sofosbuvir.

Formaldehyde was added for cell fixation, and the cells were stained using the anti-serum of patients infected by hepatitis C virus, HRP-goat antihuman IgG, and DAB substrate kits (Thermo Fisher Scientific®). The percent inhibition and IC_{50} value were calculated by counting the brown color of infected cells using a microscope [24,25].

3. Results and disscussion

3.1. In silico studies

3.1.1. Molecular docking analysis

In the in silico studies, molecular docking was employed to evaluate the interaction between test compounds and protein targets. NS5B protein (PDB ID: 3FQL) was chosen as the protein target because it serves as a main polymerase of the HCV, in which the inhibition of this enzyme will terminate the replication of the HCV [26].

Prior the docking simulation, the native ligand (HCV-796) was removed, and this molecule was re-docked to this NS5B protein. This validation aimed to ensure that the method used is acceptable and valid [27]. RMSD between the docked ligand coordinates and the crystal structure coordinates was taken as consideration for a validation process. An RMSD value below 2 Å means the molecular docking method is acceptable and valid [28]. The grid box covering the binding site of the native ligand (HCV-796) was used for all of the docking experiments.

The re-docking process showed a RMSD value of 0.98 Å with the lowest free binding energy value of −12.51 kcal/mol, revealing that the docking methods are acceptable and valid. Molecular docking between α-mangostin, β-sitosterol, piperine, and sofosbuvir with NS5B protein revealed that α-mangostin, β-sitosterol, piperine, and sofosbuvir showed a negative binding free energy of −10.16 kcal/mol, −10.87 kcal/mol, −9.37 kcal/mol, and −8.77 kcal/mol, respectively. α-mangostin and β-sitosterol showed comparable binding affinity at NS5B, while piperine and sofosbuvir showed slightly lower binding affinity at NS5B. The molecular interaction was investigated to evaluate the binding mode of the native ligand (Fig. 1a), α-mangostin (Fig. 1b), β-sitosterol (Fig. 1c), piperine (Fig. 1d), and sofosbuvir (Fig. 1d) with NS5B protein (see also Fig. 2) with BIOVIA discovery Studio® software.

From Figs. 1a and 2, it can be seen that TYR415, TYR448, ARG200, SER368, PRO197, LEU384, LEU204, VAL321, ILE363, LEU204, SER365, ASP319, CYS366, CYS316 and PHE193 mediated the interaction between native ligand and NS5B protein. The interaction between α-mangostin and NS5B protein (Figs. 1b and 2) were mediated by SER365, SER368, ARG200, CYS316, CYS366, VAL321, LEU204, ILE363, LEU360, VAL370, ILE323, TYR415, LEU384, MET414, PRO197, TYR448, in which the hydrogen bonding and hydrophobic interaction were feasible. The interaction between β-sitosterol and NS5B protein (Figs. 1c and 2) were mediated by MET414, LEU384, ILE363, LEU360, VAL370, LEU204, VAL321, LEU314, CYS366, CYS316. The hydrophobic interaction played a crucial role in the interrelation between β-sitosterol and NS5B protein. The interaction between piperine and NS5B protein (Figs. 1d and 2) were mediated by VAL370, LEU360, LEU204, TYR415, PRO197, MET414, CYS366, VAL321, LEU384, ILE363. The interaction between sofosbuvir and NS5B protein were mediated by ILE363, SER365, TYR448, TYR555, CYS316, VAL370, ARG200, MET414, LEU314, LEU204, VAL321, CYS366, LEU384, PHE193, PRO197.

It can be seen that the native ligand (HCV-796), α-mangostin, β-sitosterol, piperine, and sofosbuvir interacted with NS5B protein through similar amino acids: ILE363, LEU204, VAL321, CYS366, and LEU384. This might imply that these interactions played important roles in antiviral activity of these ligands.

3.1.2. Molecular dynamics simulation analysis

The best conformation of the test compounds, determined through molecular docking at the NS5B protein, was further analyzed using molecular dynamics simulation for a duration of 200 ns (Fig. 3a–f).

The RMSD-graph of molecular dynamics simulation (Fig. 3a) showed the RMSD value less than 3 Å for all of the test compounds

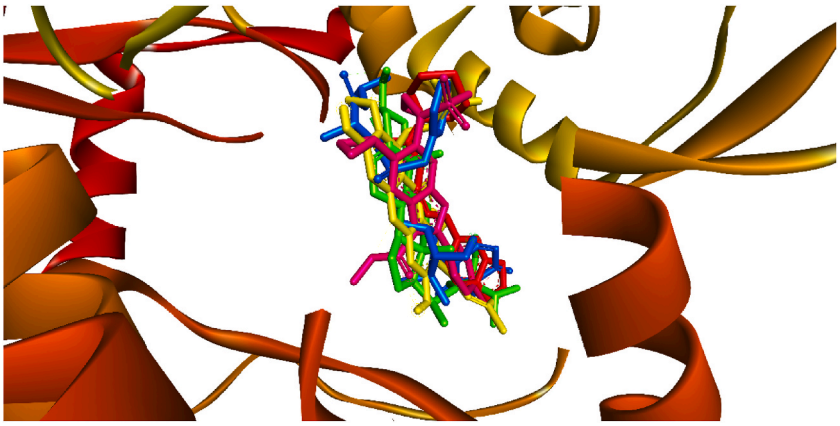

Fig. 2. Visualization of 3D (three-dimensional) of molecular docking interaction of ligands with NS5B protein (PDB ID 3FQL): Native ligand (pink), α-mangostin (yellow), β-sitosterol (green), piperine (red), and Sofosbuvir (blue).

(native ligand, α-mangostin, β-sitosterol, piperine, and sofosbuvir) with NS5B protein. This indicates there a lack of conformational changes of the ligand inside the determined binding pocket of NS5B protein [29]. The average RMSD value of the native ligand complex with NS5B protein was 1.87 Å, the average RMSD value of α-mangostin with NS5B protein was 1.98 Å, the average RMSD value of native β-sitosterol with NS5B protein was 2.08 Å, the average RMSD value of piperine with NS5B protein was 1.90 Å and the

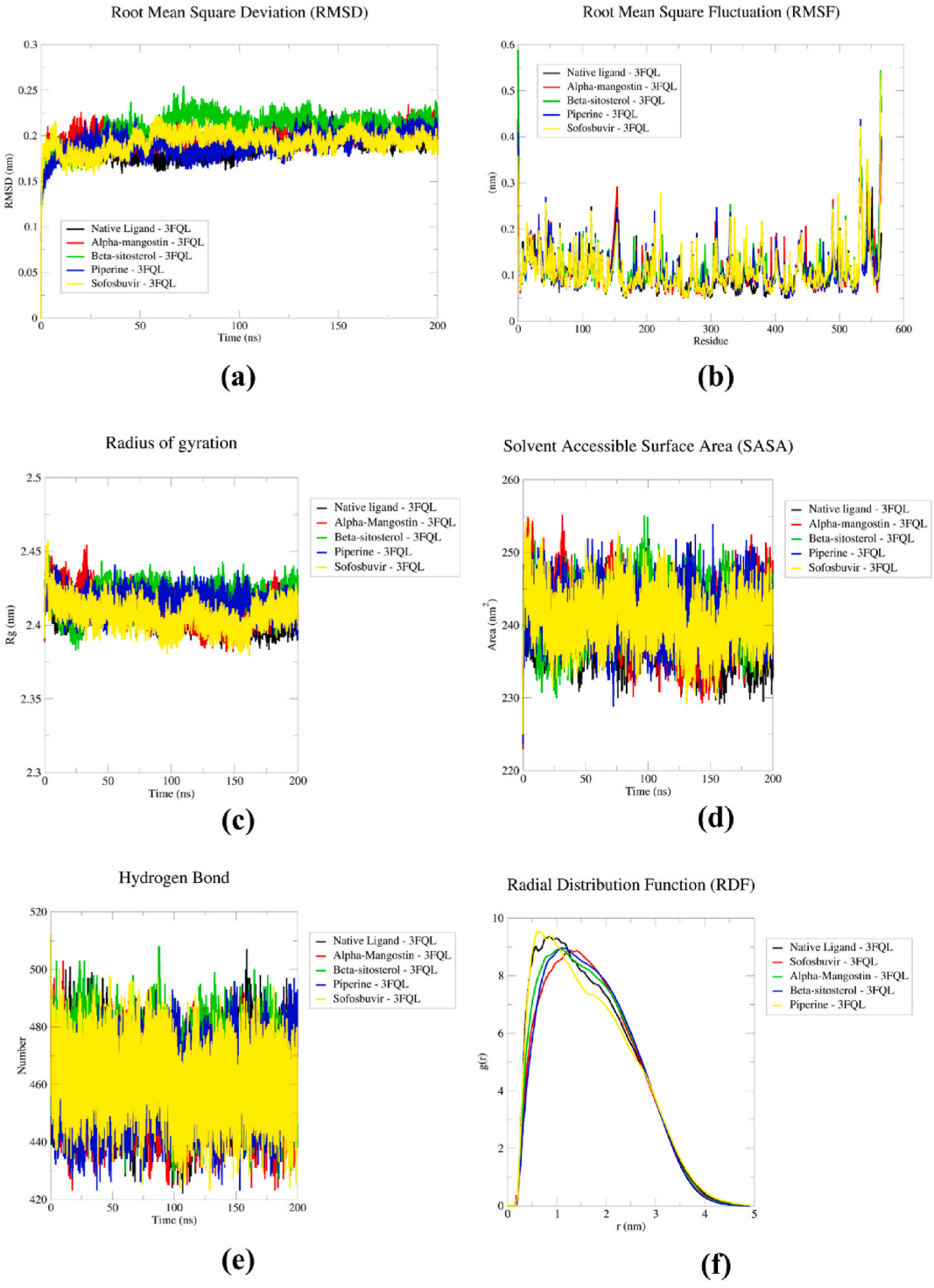

Fig. 3. Grapics of (a) RMSD, (b) RMSF, (c) Radius of gyration, (d) SASA, (e) Hydrogen Bond, and (f) RDF during molecular dynamics simulation of the test compounds at NS5B protein.

average RMSD value of sofosbuvir with NS5B protein was 1.93 Å.

The RMSF-graph, which exhibits the flexibility of amino acid residues during the simulation periods, was also evaluated (Fig. 3b). The average RMSF value of native ligand, α-mangostin, β-sitosterol, piperine, and sofosbuvir complex with NS5B protein was 1.08 Å, 1.10 Å, 1.16 Å, 1.12 Å and 1.12 Å, respectively. This might indicate a lack of amino acids movement, which may be caused by the stabilization of the compound inside the binding pocket of the protein. Additional analysis with a gyration graph showed a similar effect, suggesting that all complexes were stable during molecular dynamics simulation.

In order to check the capability of the water molecule to enter the complexes, solvent-accessible surface area (SASA)-graphs were generated (Fig. 3d). The average SASA value of the native ligand complex with NS5B protein was 239.56 nm^2. In comparison, the average SASA values for complexes with α-mangostin, β-sitosterol and piperine complex was 241.13 nm^2, 241.62 nm^2, 241.58 nm^2, respectively. The average SASA value of sofosbuvir with NS5B protein was 240.99 nm^2. There were no significant differences between the SASA-graph from all test compounds. Thus, it can be assumed that all complexes were stable during molecular dynamics simulation accessible to water molecules.

Then an evaluation of radial distribution function (RDF) and hydrogen bond stability (H-bond) during molecular dynamics simulations were performed. Based on the RDF and H-Bond graphs, there were no significant differences between their RDF and H-Bond graphs, which in agreement with the SASA-graph results.

Finally, based on the binding free energies calculation results by using MM-PBSA and MM-GBSA methods. NS5B-α-mangostin complex resulted the free energy binding of −9.13 kcal/mol (MM-PBSA calculation) and −49.09 kcal/mol (MM-GBSA calculation) while NS5B-β-sitosterol complex resulted the free energy binding of −0.05 kcal/mol (MM-PBSA calculation) and −54.66 kcal/mol (MM-GBSA calculation), NS5B-piperine complex resulted the free energy binding of −2.36 kcal/mol (MM-PBSA calculation) and −42.30 kcal/mol (MM-GBSA calculation), NS5B-sofosbuvir complex resulted the free energy binding of 2.58 kcal/mol (MM-PBSA calculation) and −53.24 kcal/mol (MM-GBSA calculation) and NS5B-native ligand complex resulted the free energy binding of −70.85 kcal/mol (MM-PBSA calculation) and −18.85 kcal/mol (MM-PBSA calculation). NS5B-α-mangostin-complex showed more stable binding complex formation compared to other phytochemical-NS5B complexes.

3.1.3. Analisis of ADMET predition

ADMET prediction was performed by inputting the structure of the compounds in SMILES format; then, the application would predict the compounds' pharmacokinetic parameters and toxicology information based on the compounds' molecular properties and

Table 1
ADMET prediction the compounds based on ADMET predictor of pkCSM online.

Absorption Parameters	α-mangostin	β-sitosterol	Piperine	Sofosbuvir
Water Solubility (log mol/L)	−4.067	−6.773	−3.464	−3.953
Caco-2 Permeability (Good if the values > 0.9 log Papp in 10^{-6} cm/s)	−0.048	1.201	1.596	0.472
Intestinal absorption (human) (% Absorbed)	93.647	94.464	94.444	64.308
Skin Permeability (log Kp)	−2.736	−2.783	−3.131	−2.736
P-glycoprotein substrate	Yes	No	Yes	Yes
P-glycoprotein I inhibitor	Yes	Yes	Yes	Yes
P-glycoprotein II inhibitor	Yes	Yes	No	No
Distribution Parameters				
VDss (human) (log L/kg)	−0.282	0.193	0.158	−0.728
Fraction unbound (human) (Fu)	0	0	0.134	0.08
BBB permeability (log BB)	−1.075	0.781	−0.102	−1.873
CNS permeability (log PS)	−1.984	−1.705	−1.879	−4.343
Metabolism Parameters				
CYP2D6 substrate	No	No	No	No
CYP3A4 substrate	Yes	Yes	Yes	No
CYP1A2 inhibitor	Yes	No	No	No
CYP2C19 inhibitor	Yes	No	Yes	No
CYP2C9 inhibitor	Yes	No	No	No
CYP2D6 inhibitor	No	No	No	No
CYP3A4 inhibitor	No	No	No	No
Excretion Parameters				
Total Clearance (log ml/min/kg)	0.43	0.628	0.232	−0.106
Renal OCT2 substrate	No	No	Yes	No
Toxicity Parameters				
AMES toxicity	Yes	No	No	No
Max. tolerated dose (human) (log mg/kg/day)	0.061	−0.621	−0.38	1.049
hERG I inhibitor	No	No	No	No
hERG II inhibitor	Yes	Yes	No	No
Oral Rat Acute Toxicity (LD50)(mol/kg)	1.949	2.552	2.811	2.31
Oral Rat Chronic Toxicity (LOAEL)(log mg/kg_bw/day)	1.594	0.855	1.51	1.824
Hepatotoxicity	No	No	Yes	Yes
Skin Sensitization	No	No	No	No
T.Pyriformis toxicity *(log ug/L)*	0.325	0.43	1.879	0.285
Minnow toxicity (log mM)	−0.138	−1.802	1.732	1.023

functional group. The results of ADMET prediction of the compounds are shown in Table 1.

From Table 1, 7 parameters related to absorption are presented. β-Sitosterol had the lowest water solubility value (in log mol/L). Additionally, both β-sitosterol and piperine showed acceptable caco-2 permeability values. All the compounds exhibit favorable intestinal absorption in humans, as their values excedeed 30%. Each test compounds also displays commendable skin permeability, as indicated by their log Kp values being lower than −2.5 [30]. Among all the compounds, only α-mangostin is identified as substrate and inhibitor for P-glycoprotein.

The distribution consists of 4 different parameters. β-Sitosterol exibithed the highest values of the VDss in humans, CNS, and blood-brain barrier permeability compared to the others. However, piperine had the higer fraction values of unbound than α-mangostin, piperine and sofosbuvir. On the other hands, only sofosbuvir that was not as cytochrome P450 inhibitors and CYP2D6/CYP3A4 substrate in the metabolism parameters. Regarding excretion parameters, β-sitosterol had the highest value for total clearance, and only piperine acted as a renal OCT2 substrate.

Toxicity parameters consisted of 10 parameters to be evaluated. Of these, only α-mangostin tested positive for AMES toxicity. Sofosbuvir exhibited the highest value for the maximum tolerated dose in humans. Furthermore, all the compounds were not identified as hERG I inhibitors and did not cause skin sensitization. Piperine and sofosbuvir were not classified as hERG II inhibitors, yet both were toxic to the liver. Additionally, piperine displayed the highest toxicity values for T. Pyriformis and minnow. In terms of oral rat toxicity, α-mangostin was the most toxic based on acute measures, while β-sitosterol was the most toxic for chronic measures compared to the others.

3.2. In vitro anti-hepatitis C virus activity

Encouraging by the in silico results, we further study the effect of the α-mangostin, β-sitosterol, and piperine in cells based assays. Sofosbuvir, an NS5B polymerase HCV inhibition, that serve as positive control was shown to have the IC_{50} of 0.06 ± 1.76 μM, indicating the antiviral activity of sofosbuvir at the HCV. The IC_{50} value of α-mangostin was 2.70 ± 0.92 μM that was consistent with the results of in silico studies. Surprisingly, the IC_{50} of piperine was 52.18 ± 3.21 μM, while the IC_{50} of β-sitosterol was >100 μM. Those results indicated that piperine possessed moderate activities while β-sitosterol did not show any activity in the tested doses. These results may not provided parallel activities with the in silico (molecular docking) studies of ligand-protein interaction. The molecular docking simulation was conducted under vacuum condition, without considering temperature, time and solvent parameters. In contrast, the cell-based assays were performed in an environment that is significantly more complex. Further studies are needed to evaluate the detailed molecular mechanism of these test compounds. Although in this study the IC_{50} value of α-mangostin compound was 45 times and piperine was more than 869 times compared to the IC_{50} of sofosbuvir, these compounds hopefully could be developed in further research to become alternative of anti-HCV from plants.

4. Conclusion

In conclusion, phytochemical α-mangostin, β-sitosterol and piperine were evaluated with the aim to investigate the molecular mechanism of these phytochemical as anti-HCV. The α-mangostin showed the lowest free binding energy using MM-PBSA calculation (−9.13 kcal/mol), while β-sitosterol was −0.05 kcal/mol and piperine was −2.36 kcal/mol, so that α-mangostin and piperine interacted better than β-sitosterol at the protein target. Additionally, the cell-based assay showed an inhibitory activity of α-mangostin with the IC_{50} value of 2.70 ± 0.92 μM at hepatocarcinoma cell lines. However, this in vitro test did not specifically indicate whether the compound inhibited NS5B protein. Although further in vitro research in specific protein target (NS5B protein) is needed, α-mangostin can act as valuable lead compound for further research of the anti HCV.

Author contribution statement

Anjar Hermadi Saputro: Wrote the paper; Performed the experiments; Analyzed and interpreted the data. Aty Widyawaruyanti, Tutik Sri Wahyuni: Wrote the paper; Performed the experiments; Conceived and designed the experiments. Adita Ayu Permanasari: Wrote the paper; Performed the experiments. Aluicia Anita Artarini, Daryono Hadi Tjahjono, Andhika Bintang Mahardhika, Tasia Amalia: Wrote the paper; Analyzed and interpreted the data. Sophi Damayanti: Wrote the paper; Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools, or data.

Data availability statement

Data will be made available on request.

Funding statement

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Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

ACPYPE AnteChamber Python Parser interfacE
DMEM Dulbecco's Modified Eagle Medium
HCV Hepatitis C Virus
MM-GBSA Molecular mechanics generalized Born surface area
MM-PBSA Molecular mechanics Poisson–Boltzmann surface area
MOI multiplicity of infection
PDB ID Protein data bank ID
RDF radial distribution function
RMSD root mean square deviation
RMSF root mean square fluctuation
SASA solvent-accessible surface area

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