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Antiviral and molecular docking analysis of methoxyflavones isolated from *Melicope latifolia* leaves against HCV

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ABSTRACT: Treatments of hepatitis C virus have been developed and increased the sustained virology response (SVR), however, there are several reports of drug resistance, high-cost issue, and limited access to current hepatitis C virus (HCV) treatment that remain become a problem. This necessitates to search for complementary and alternative drugs against HCV, therefore the investigation of active compounds from plant extracts such as *Melicope latifolia*, a plant that has been reported as anti-HCV, will be provided in this study. The anti-HCV activities were tested using in vitro cultured cells of hepatocyte cell line Huh 7.5 and HCV genotype 2a (J6/JFH1). Ethanol extract of *M. latifolia* leaves was separated by chromatographic methods and the chemical structures of the isolated compounds were established based on mass spectrometry, 1D, and 2D nuclear magnetic resonance spectral data, as well as comparison with reported data. The interaction of the compound with the protein, which involves to HCV activity, was determined by docking analysis. Three known methoxyflavone compounds identified as 5,4'-dihydroxy-7-prenyloxy-3,8,5'-trimethoxyflavone (1); 5,3'-dihydroxy-3,7,8,4'-tetramethoxyflavone (2); and 5-hydroxy-3,7,8,3',4'-pentamethoxyflavone (3) were isolated from the ethanolic extract of *M. latifolia* leaves. Anti-HCV activities revealed that compound (1) strongly inhibited HCV J6/JFH1 with a 50% inhibitory concentration (IC_{50}) value of 6.7±0.4 µg/mL and 50% cytotoxic concentration (CC_{50}) of 19.3 µg/mL. The docking analysis revealed an interaction with the 4GAG, a protein that involves in the entry step of HCV, and the 4EAW protein which plays an important role during HCV replication.

KEYWORDS: AntiHCV; Infectious disease; *Melicope latifolia*; medicine; hepatitis; health

1. INTRODUCTION

Hepatitis C Virus (HCV) is a single-stranded RNA virus that belongs to the Flaviviridae family. The HCV genomes are translated into 10 mature proteins that consist of 3 structural proteins and 7 non-structural proteins. The structural protein i.e core, E1, and E2 are responsible for binding and virus infection while the non-structural protein i.e NS2, NS3, NS4A, NS4B, NS5A, and NS5B are essential for viral RNA replication [1, 2].

The prevalence of HCV infection is estimated to be 170 million cases worldwide. The current standard of care for anti-HCV is a triple combination of interferon (IFN)-α, ribavirin, and the first generation of HCV NS3 protease inhibitors (telaprevir or boceprevir). These drugs have increased the sustained virological response (SVR) rate to 70% for patients infected with HCV genotype 1. Recently, it was documented that a combination of novel direct-acting antiviral agents (DAAs) could improve the SVR rates by more than 90%. However, only a limited number of patients can access this treatment due to the high cost and resistance issues

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[3, 4]. Therefore, there is a need to search for new anti-HCV drugs for complementary and or alternative treatment of HCV infections.

Medicinal plants are potential sources of new drugs. Bioactive molecules in plants are known to exhibit beneficial pharmacological effects against pathogenic agents including HCV. Some plant extracts have been shown to inhibit HCV protease such as *Boswellia carterii* and *Embelia schimperi* [5]. Some examples of phytochemical constituents isolated from plants that have been reported to inhibit HCV include: sylimarin from *Silybum marianum*, epigallocatechin-3-gallate (EGCG) from *Camellia sinensis*, ladanein-BJ486K from *Marrubium peregrinum, L.*, narigenin from grapefruit, quercetin from *Embelia ribes*, honokiol from *Magnolia grandiflora*, 3-hydroxycaruilignan C from *Swietenia macrophylla*, and excoecariphenol D from *Excoecaria agallocha* [6-11].

Moreover, another study reported anti-HCV activity in *Glycyrrhiza uralensis* extract and its isolated compounds, glycycoumarin, glycyrin, glycyrol, and liquiritigenin, with 50% inhibitory concentration (IC_{50}) of 8.8, 7.2, 4.6 and 16.4 µg/mL, respectively [12]. The isolated compound from *Morinda citrifolia* leaves (pheophorbide A) also possesses anti-HCV activities with IC_{50} of 0.3 µg/mL [13]. The previous study showed that the ethanol extracts of *Melicope latifolia* leaves have been found to possess anti-HCV activities, with IC_{50} of 3.5 µg/mL against the HCV J6/JFH1-P47 and 2.1 µg/mL against HCV J6/JFH1-P1 strains [14].

In order to isolate the active compound from *M. latifolia*, this study performed bioactivity-guided fractionation of *Melicope latifolia* syn.: *Euodia latifolia* DC (Rutaceae), commonly known as *ki sampan* in Indonesia. It is worthwhile to mention that *Melicope* species are characterized by the presence of methoxylated flavonoids that are reported to have various effects on several diseases [15-18].

2. RESULTS

2.1. Chemical Structure of Isolated Compounds

To obtain the isolated compound from *M. latifolia*, bioassay guided isolation was performed and resulted three known methoxyflavone. The structural determinations of isolated compounds were identified by LC-MS/GC-MS, ^{1}H-NMR, and ^{13}C-NMR as well as comparison with reported data (Figure 1).

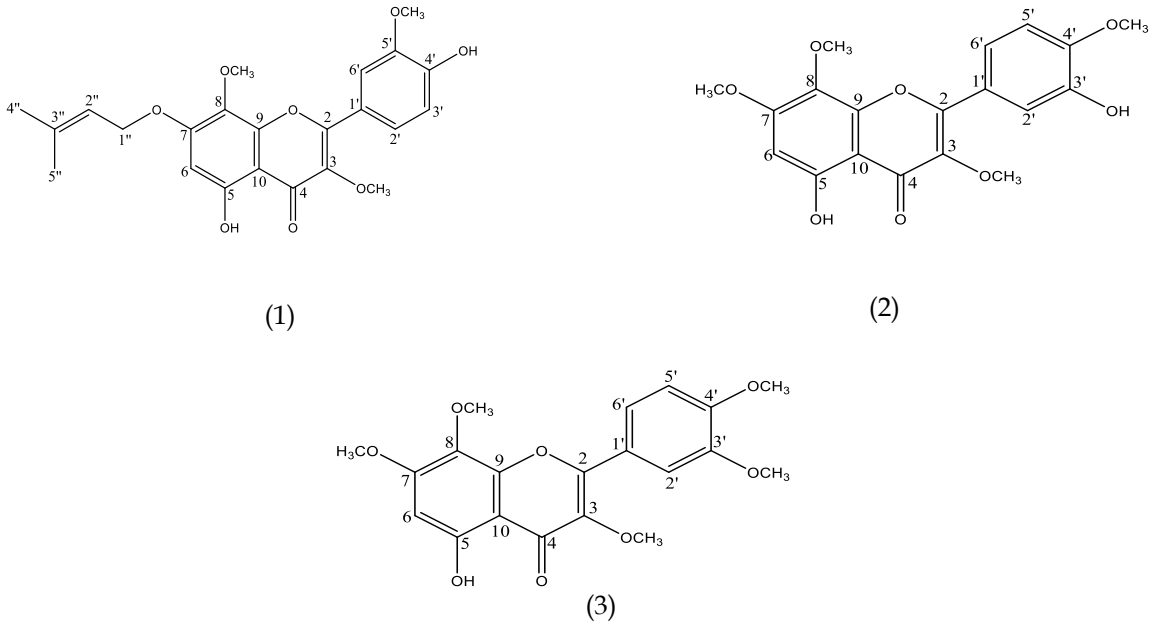

Figure 1: Molecular structure of flavonoids compounds isolated from *Melicope latifolia* leaves. Compound 1 was identified as 5,4′-dihydroxy-7-prenyloxy-3,8,5'-trimethoxyflavone (1); Compound 2 was identified as 5,3′-Dihydroxy-3,7,8,4′-tetramethoxyflavone (2); and compound 3 was identified as 5-hydroxy-3,7,8,3′4′-pentamethoxyflavone (3).

2.1.1. Compound 1 [19].

Compound 1 was identified as 5, 4′-dihydroxy-7-prenyloxy-3,8,5'-trimethoxyflavone (1): Compound 1 is a yellow amorphous powdery substance, HR-ESI-MS (negative mode) *m/z*: 427.1394 ([M-H]-); EI-MS *m/z* (rel. int. %): 428 [M^+] (100), 359 [M^+ –CH_2–CH=C (CH_3)$_2$] (80), 344 (50%), 329 (5) corresponding with the molecular formula of $C_{23}H_{24}O_8$. UV λ_{max} nm (mAU): 363 (50); 273 (57) and 258 (57). The ^{1}H-NMR spectral data were identical with those in ref: ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 3.81, 3.91, 4.06 (each 3H, s, OCH_3), 6.79 (1H, s, H-6), 7.36 (1H, d, *J* = 8.4, H-3′), 8.07 (1H, dd, *J* = 8.4, 2.0 Hz, H-2′), 7.77 (1H, d, J=1.8 Hz, H-6′), 4.64 (2H, d, J=8.5 Hz, H-1′′), 5.48 (1H, t, J=8.2 Hz, H-2′′), 1.78 (3H, s, H-4′′), 1.75 (3H, s, H-5′′), 13.15 (1H, s, OH-5). ^{13}C-NMR (DMSO-$_{d6}$, 125 MHz): 155.6 (C2), 138.5 (C3), 178.9 (C4), 157.7 (C5-OH), 96.5 (C6), 157.0 (C7), 129.0 (C8), 105.2 (C10), 122.6 (C1′), 122.8 (C2′), 114.7 (C3′), 148.4 (C4′-OH), 146.4 (C5′), 110.7 (C6′), 66.1 (C1′′), 118.8 (C2′′), 138.9 (C3′′), 25.8 (C4′′), 18.3 (C5′′), 60.1 (C3-OCH_3), 61.4 (C8-OCH_3), 55.9 (C5′-OCH_3).

2.1.2. Compound 2 [20].

Compound 2 was identified as 5, 3′-dihydroxy-3,7,8,4′-tetramethoxyflavone (2): Compound 2 is a yellow powdery substance; GCMS-QP DI-Mass (positive mode) *m/z*: 374 ([M-H]$^+$) corresponding with the molecular formula of $C_{19}H_{18}O_8$. The ^{1}H-NMR spectral data were identical with those in ref: ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 3.81, 3.82, 3.86, 3.92 (each 3H, s, OCH_3), 6.60 (1H, s, H-6), 7.00 (1H, d, *J*=8.9 Hz, H5′), 7.64 (1H, dd, *J*=8.5, 2.0 Hz, H-6′), 7.67 (1H, d, *J*=2.1 Hz, H-2′). ^{13}C-NMR (DMSO-$_{d6}$, 125 MHz): 155.7 (C2), 137.7 (C3), 178.3 (C4), 156.4 (C5-OH), 95.8 (C6), 158.2 (C7), 128.3 (C8), 147.8 (C9), 104.5 (C10), 122.3 (C1′), 115.9 (C2′), 147.5 (C3′-OH), 150.1 (C4′), 111.6 (C5′), 120.7 (C6′), 59.8 (C3-OCH_3), 56.6 (C7-OCH_3), 61.1 (C8-OCH_3), 55.5 (C4′-OCH_3).

2.1.3. Compound 3 [21].

Compound 3 was identified as 5-hydroxy-3,7,8,3′4′-pentamethoxyflavone (3): Compound 3 is a yellow powdery substance, GCMS-QP DI-Mass (positive mode) *m/z*: 388 ([M-H]$^+$), corresponding with the molecular formula of $C_{20}H_{20}O_8$. The ^{1}H-NMR spectral data were identical with those in ref: ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 3.83, 3.83, 3.85, 3.87, 3.92 (each, 3H, s, OCH_3), 6.61 (1H, s, H-6), 7.22 (1H, d, *J*=8.7 Hz, H-5′),7.67 (1H, d, *J*=2.3 Hz, H-2′), 7.74 (1H, dd, *J*=2.2, 8.7 Hz, H-6′) and 12.40 (1H, s, OH-5). ^{13}C-NMR (DMSO-$_{d6}$, 125 MHz): 155.8 (C2), 138.5 (C3), 178.8 (C4), 156.9 (C5-OH), 96.3 (C6), 158.7 (C7), 128.8 (C8), 148.3 (C9), 105,0 (C10), 122.7 (C1′), 112.2 (C2′), 148.9 (C3′), 151.8 (C4′), 111.3 (C5′), 122.4 (C6′), 60.3 (C3-OCH_3), 56.2 (C7-OCH_3), 61.5 (C8-OCH_3), 57.0 (C3′-OCH_3), 55.9 (C4′-OCH_3).

2.2. Anti-HCV and toxicity assay

An ethanol extract from *M. latifolia* leaves showed anti-HCV activity with IC_{50} value of 3.5 ± 1.4 µg/mL and CC_{50} >100 µg/mL. These results suggest that ethanol extract containing active compounds from *M. latifolia* leaves possesses strong anti-HCV activity that is not mediated by a cytotoxic effect (SI=Selectivity index >28.6). The dichloromethane fraction also showed strong anti-HCV activity, with an IC_{50} value being 1.7±0.4 µg/mL and CC_{50}>30 µg/mL. Further separation of dichloromethane fraction was conducted by Vacuum Liquid Chromatography (VLC) and yielded 9 subfractions (D1 - D9). Anti-HCV testing of these nine subfractions revealed that subfraction D3 had a strong anti-HCV activity with IC_{50} value of 2.4±0.3 µg/mL. Subfraction D3 was then further subjected to open column chromatography on Sephadex LH-20 and yielded 7 subfractions (D3.1 - D3.7). To demonstrate the anti-HCV activities of 7 subfractions from D3, a further 4 active subfractions were obtained. D3.3; D3.4; D3.5 and D3.6 were all found to have anti-HCV activity with IC_{50} values of 0.2±0.2; 0.8±0.4; 3.6±1.1 and 0.6±0.2 µg/mL, respectively. Subfraction D3.3 exhibited the highest activity without any cytotoxicity effect. Thus, further analysis was performed on this subfraction. The purification of subfraction D3.3, D3.4, D3.5 and D3.6 yielded compounds 1, 2, and 3, which were identified as 5,4'-dihydroxy-7-prenyloxy-3,8,5′-trimethoxyflavone (1), 5,3'-dihydroxy-3,7,8,4'-tetramethoxyflavone (2) and 5-hydroxy-3,7,8,3',4'-pentamethoxyflavone (3) as Figure 1. Compound 1 exhibited strong anti-HCV activity on the post J6/JFH1 infection of Huh7.5 cells with the IC_{50} value of 6.7±0.4 µg/mL while compound 2 and 3 have IC_{50} > 30 µg/mL (Figure 2). The 50% inhibitory concentration (IC_{50}), 50% cytotoxicity concentration (CC_{50}), and selective index (SI) of the *M. latifolia* extracts presented in this study are shown in Table 1.

Table 1: The anti HCV activity of extract/fraction/subfraction and constituents of *M.Latifolia*.

Substances	IC_{50} (µg/mL)	CC_{50} (µg/mL)	SI(CC_{50}/ IC_{50})
Ethanol extract	3.5 ± 1.4	>100	>28.6
Dichloromethane fraction (D)	1.7 ± 0.4	>30	>17.6
Dichloromethane subfraction (D3)	2.4 ± 0.3	>30	>12.5
Dichloromethane subfraction (D3-3)	0.2 ± 0.2	>100	>500
Dichloromethane subfraction (D3-4)	0.8 ± 0.4	50.2	62.8
Dichloromethane subfraction (D3-5)	3.6 ± 1.1	39.6	11
Dichloromethane subfraction (D3-6)	0.6 ± 0.2	44.1	73.5
Compound 1	6.7 ± 0.4	19.3	2.9
Compound 2	>30	>20	n.a
Compound 3	>30	>50	n.a

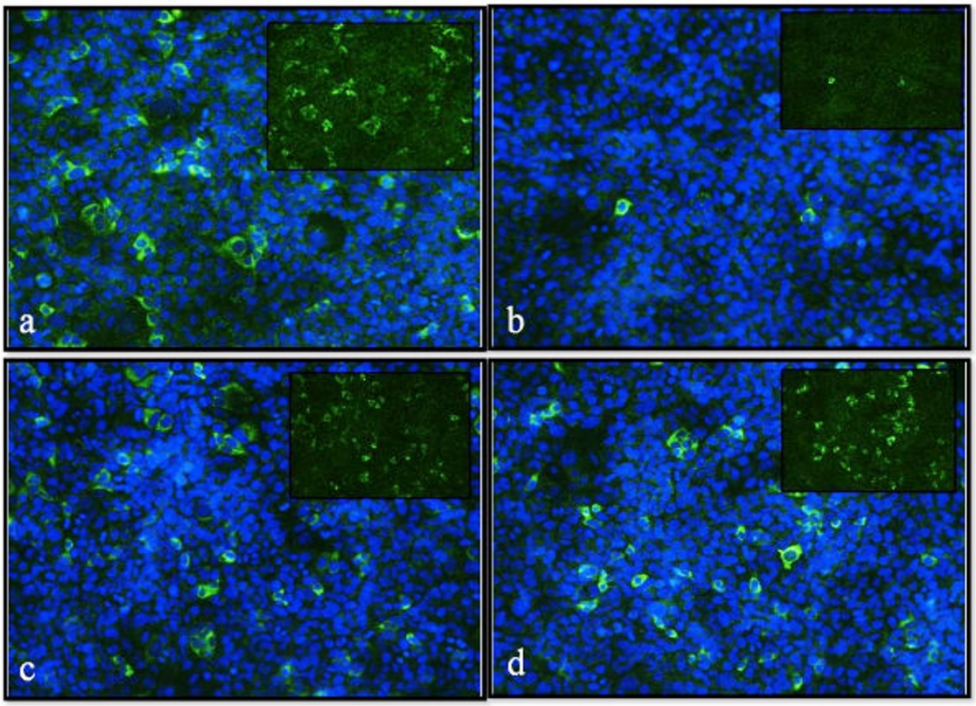

Figure 2: Immunofluorescent analysis (IFA). (a) The untreated control (0.1% DMSO). (b) 5,4′-dihydroxy-7-prenyloxy-3,8,5'-trimethoxyflavone (compound 1). (c); 5,3′-Dihydroxy-3,7,8,4′-tetramethoxy-flavone (compound 2). (d) 5-hydroxy-3,7,8,3′4′-pentamethoxyflavone (compound 3).The structural determinations of isolated compounds were identified by LC-MS, ^{1}H-NMR and ^{13}C-NMR.

2.3. Immunofluorescence analysis (IFA)

The infected cells were detected by FITC immunostaining which demonstrated the infected cells as green fluorescence. The results showed that compound 1, 5,4'-dihydroxy-7-prenyloxy-3,8,5′-trimethoxyflavone less infectivity compared to controls. In contrary, there was no inhibition found in compound 5,3'-dihydroxy-3,7,8,4'-tetramethoxyflavone (compound 2); and 5-hydroxy-3,7,8,3',4'-pentamethoxyflavone (compound 3) (Figure 2).

2.4. Mode-of-action of inhibition

Time-of-drug addition experiments were conducted to determine which step of the HCV life cycle was inhibited by compound 1. The results showed that compound 1 mainly inhibited the virus at the entry step with a 70% reduction of HCV infectivity in the culture supernatants compared to the untreated control. On the other hand, treatment at the post-entry stage showed a 20% reduction of HCV infectivity compared to the untreated control. The treatment of cells with compound 1 in the whole viral stage (entry and post-entry steps) showed the highest inhibition percentage (Figure 3).

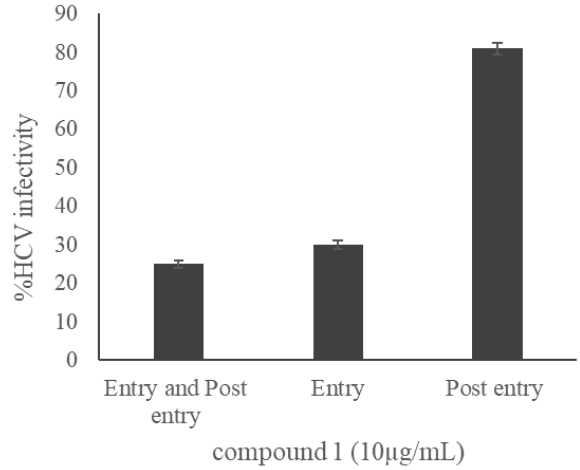

Figure 3: Mode-of-action of compound 1. (Entry and Post entry): The mixture of virus and compound 1 (10 µg/mL) were inoculated to Huh 7.5 cells. After virus adsorption for 2 hrs., Huh 7.5 was cultured with the same concentrations of compound 1 for 46 hrs; At the entry step, compound 1 was added only during virus inoculation for 2 hrs; At the post-entry stage, cells were infected with virus in the absence of compound 1. After viral absorption, the cells were cultured with compound 1-containing medium for 46 hrs.

2.5. Docking analysis

To predict the interaction of the active compound to the receptor, a docking analysis was performed. The Rerank Scores were shown in Table 2 and 3. The result was obtained a strong interaction to the 4GAG.pdb, a protein that involves in the entry step of HCV. The 4GAG receptor neutralized antibody AP33 in complex with its HCV Epitope E2 (Figure 4 and 5). Moreover, compound 1 gave an interaction to 4EAW.pdb, a protein of HCV NS5B that involved a replication step of HCV life cycle (Figure 6). The 3D interaction was clearly described in Figure 7.

Table 2: Docking results of compound 1 on 4EAW receptors

Compounds	Rerank Score (kcal/mol)	Interaction between Ligand-Amino acids	
	4EAW	**Hydrogen-Bond**	**Steric-Interaction**
1	-98.9048	Tyr316, Asp319, Tyr448, Cys366	Tyr316, Asp319, Tyr448, Cys366, Tyr415, Phe193, Asp318
Native Ligand 0NQ_601 (A)	-93.6391	Tyr448, Asp318, As291	Tyr448, Asp318, Asn291

Table 3: Docking results of compound 1 on 4GAG receptors

Compounds	Rerank Score (kcal/mol)	Interaction between Ligand-Amino acids	
	4GAG	**Hydrogen-Bond**	**Steric-Interaction**
1	-88.4954	Gly162	Gly162, Val163, Asp167, Asp165, His164
Native Ligand GOL_302 (H)	-45.0229	Asp167, His164	Asp167

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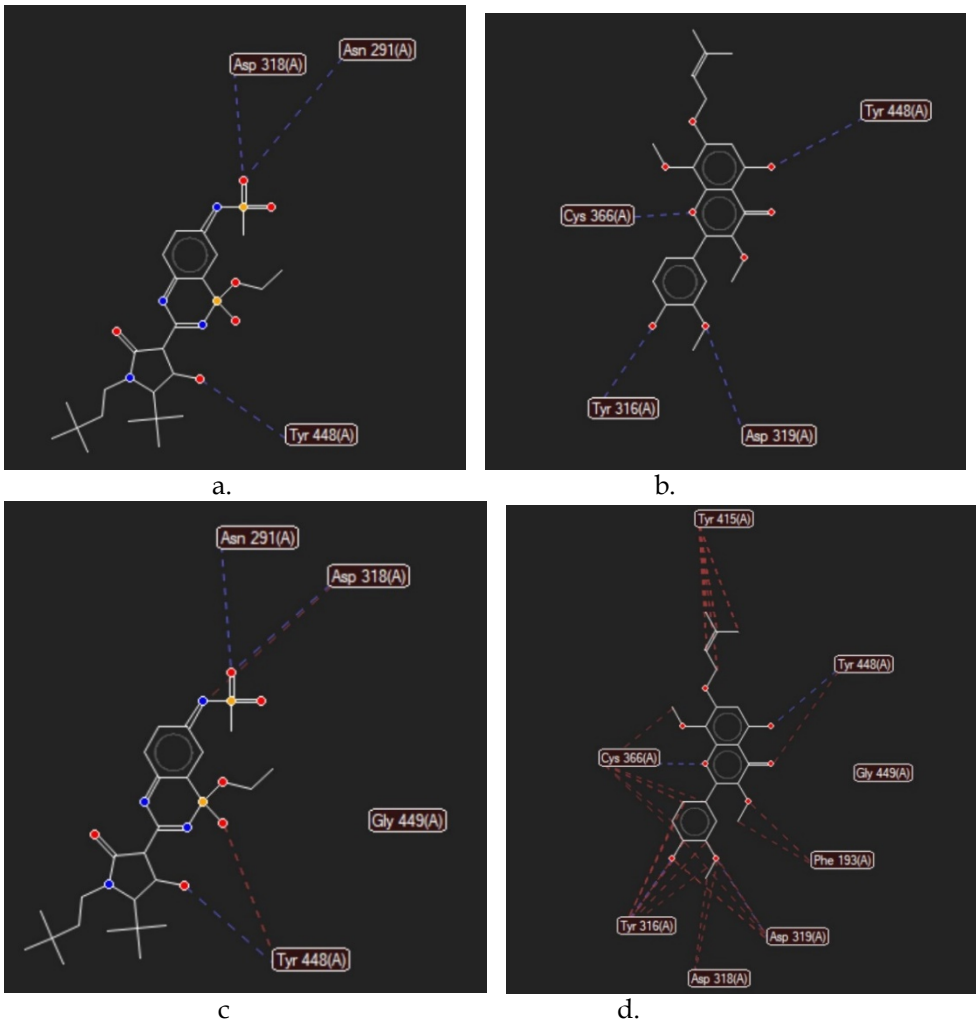

Figure 4: Hydrogen bond interaction (dashed blue-line) and Steric-Van der Waals bond interaction (dashed red-line) between Standard Ligand (a and c) and compound 1 (b and d) on the active site of HCV protein (4EAW.pdb)

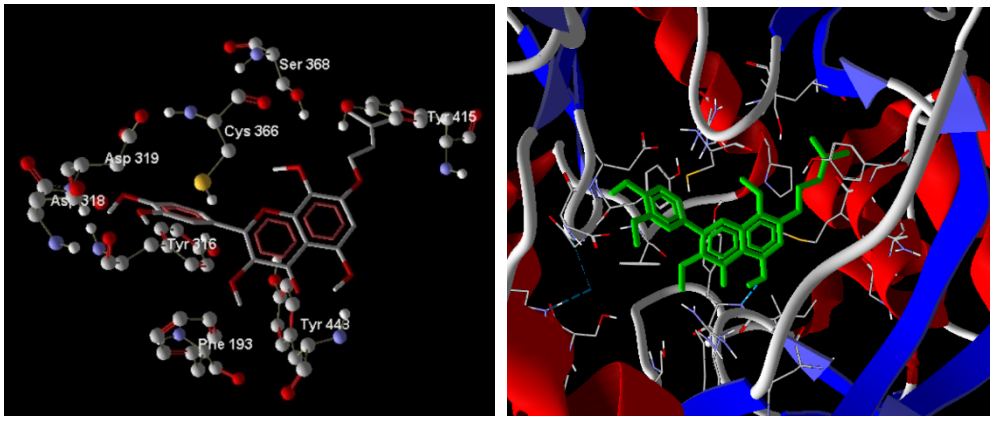

Figure 5: The 3D profile interaction of compound 1 with receptor (4EAW.pdb)

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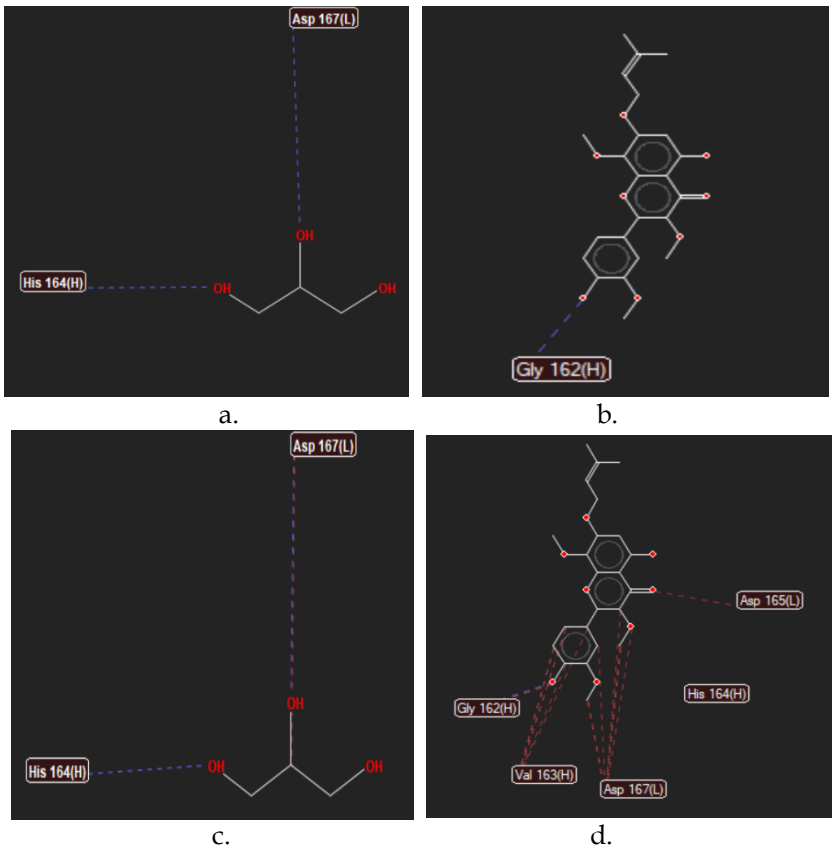

Figure 6: Hydrogen bond interaction (dashed blue-line) and Steric-Van der Waals bond interaction (dashed red-line) between Standard Ligand (a and c) and compound 1 (b and d) on the active site of HCV protein (4GAG.pdb). Compound 1 revealed a strong interaction with a rerank value of -88.4954 kcal/mol, while ligand possessed a rerank value of -45.0229 kcal/mol. These results revealed a strong binding interaction of compound 1 to 4GAG which is supported the in vitro assay of anti-HCV analysis.

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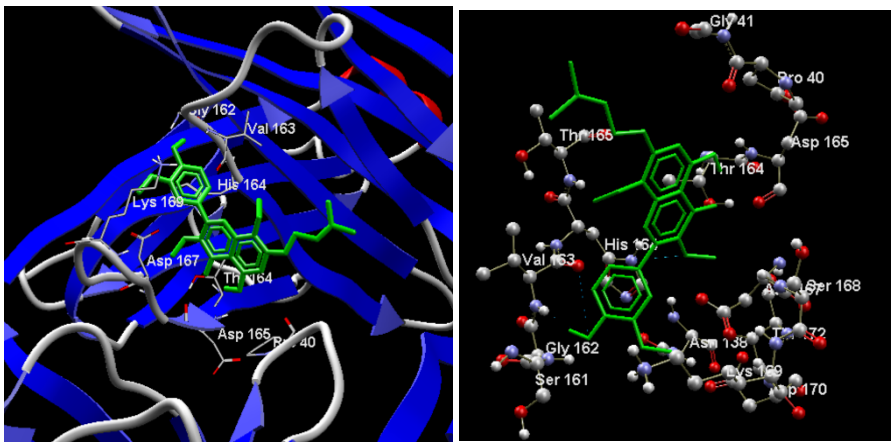

Figure 7: The 3D profile interaction of compound 1 with receptor (4GAG.pdb)

3. DISCUSSION

The chemical structures of compounds played an important role in anti-HCV activities. All three isolated compounds were methoxyflavones group which possessed a similar structure. This study obtained that only compound 1 has potential anti-HCV. The O-prenylated substitution in C-7 of flavonoid ring system of compound 1 distinguished it from the other two compounds. The prenyl group has also been found to affect the biological activity of isolated compounds. Prenylated flavonoids are an important class of phenolics, which combine a flavonoid skeleton with a lipophilic prenyl side chain. Approximately one thousand prenylated flavonoids have been identified from plants and C-prenylation on flavonoids is much more common than O-prenylation. Prenylated flavonoids are generally more active than their non-prenylated precursors. Prenylation in the flavonoid ring system could increase the lipophilicity of flavonoids, which results in a higher affinity towards biological membranes and improve interaction with target proteins, thereby resulting in the enhancement of anti-HCV activity. It is generally agreed that at least one phenolic hydroxyl group and a certain degree of lipophilicity are required for the activity of flavonoids compounds [22-24]. The anti-HCV activity of compound 1 was possible due to the presence of its prenyloxy group moeity.

The compound 1 was found to have inhibition at the virus entry and post-entry step (Figure 3). HCV entry represents the beginning of viral infection, which includes the initial recruitment and attachment of the virion to Huh7.5, post-binding interactions with host entry factors, clathrin-mediated endocytosis, and membrane fusion to release viral RNA into the cytosol. The blocking of viral entry can efficiently eradicate HCV infection at the very first step, before viral genomes start to emerge, and might prevent cell-to-cell transmission, which is also required for viral spread [25]. In the other hand inhibition at post entry step against HCV suggesting an effect on virus genome replication and assembly [27]

The result of docking analysis showed an interaction of compound 1 to 4EAW.pdb, a protein of HCV NS5B that involved a post-entry step of HCV life cycle on virus replication. The rerank score of compound 1 was -98.9048 kcal/mol while the ligand was -93.6391 kcal/mol. The lower value of rerank score indicates a better interaction with the receptor and higher binding stability between compound and receptor. It resulting in a better biological activity of compound compared to the ligand. The binding was demonstrated by hydrogen and steric van der Waals interaction to the receptor.

Compound 1 was demonstrated an hydrogen binding with Tyr316, Asp319, Tyr448, Cys366 and van der Waals interaction of Tyr316, Asp319, Tyr448, Cys366, Tyr415, Phe193, Asp318 while the standard ligand revealed a hydrogen binding interaction to Tyr448, Asp318, As291 and van der walls binding interaction to Tyr448, Asp318, Asn291 (Figure 4 and 5). Moreover, compound 1 gave strong interaction to the 4GAG, a protein that involves in the entry step of HCV. The 4GAG receptor neutralized antibody AP33 in complex with its HCV Epitope E2. The hydrogen bond interaction of compound 1 binds to Gly162, while the ligand showed a binding interaction to Asp167, His164. On the other hand, the van der Waals bond was revealed an interaction to Gly162, Val163, Asp167, Asp165, His164 of compound 1 and Asp167 of ligand (Figure 6 and 7).

Based on the docking analysis was demonstrated the interaction of phenyloxy group with Try415 at 4EAW receptor though steric-van der walls bond. It indicated that this moeity contributed an important role

in anti-HCV activity. While this compound also possesed an interaction in 4GAG receptor which involved in the entry step of HCV.

4. CONCLUSION

A methoxyflavone isolated from *M. latifolia* namely 5,4'-dihydroxy-7-prenyloxy-3,8,5'-trimethoxyflavone (compound 1) strong inhibited HCV J6/JFH1 with IC_{50} value of 6.7±0.4 µg/mL and CC_{50} of 19.3 µg/mL. HCV inhibition of this compound occurred at entry and post-entry step with a strong interaction to 4EAW and 4GAG receptor. Further analysis will be needed to elucidate the mechanism of this inhibition. These results suggested that compound 1 was potential to be developed as anti-HCV agents.

5. MATERIALS AND METHODS

5.1. General

NMR, JEOL ECA 500 spectrometer; MS, ESI-MS: LTQ Orbitrap XL and DI-Mass Shimadzu, GCMS-QP 5000/QP 5050A; UV spectra were obtained from HPLC runs with a Shimadzu SPD-M10A diode array detector. The HPLC system also included two LC-10AD pumps and a SCL-10A controller. An Imtakt Unison UK C-18 column (3 µm, 250 mm x 10 mm) was eluted with CH_3CN-H_2O containing 0.03% TFA at 0.8 ml/min (linear CH_3CN gradient from 40 to 80% in 60 min). Vacuum Liquid Chromatography (VLC) and open column liquid chromatography separations were both performed using Silica gel 60 (0.063-0.200 mm) (E. Merck). Thin Layer Chromatography (TLC) was carried out on silica gel 60 F254 and RP silica gel (E.Merck).

5.2. Plant material

Leaves of *Melicope latifolia* were collected in April 2010 and November 2011 at Cangar Forest, Batu-Malang, Indonesia and identified by the botanist in Purwodadi Botanical Garden, Indonesian Institute of Science, East Java, Indonesia with determination letter number No.0340/IPH.06/HM/III/2017.

5.3. Extraction and isolation of *Melicope latifolia* leaves

The dried powder of *M. latifolia* leaves (250 g) was extracted using *n*-hexane, which resulted in the formation of a semi-solid substance (1.7g). The residue was further extracted with 80% ethanol to obtain a crude ethanol extract (133 g), which was then partitioned in a separating funnel using a solvent of dichloromethane and water (1:1). The dichloromethane fraction (Fraction D) was subjected to vacuum liquid chromatography (VLC) over silica gel, using a gradient solvent of chloroform-methanol 100% - 97%. Nine subfractions were obtained from this separation i.e D1-D9. Subfraction D3 was separated further by open column chromatography on Sephadex LH-20, and $CHCl_3$-MeOH (5/95 % v/v) as a solvent system. Seven subfractions were collected from D3 separation (D3.1-D3.7). Subfractions D3.3, D3.4, D3.5, and D3.6 were then separated and purified by HPLC using a RP-18 column with a gradient of CH_3CN-MeOH (40% - 80%), Rt: 60 min, Flow rate: 2.5 mL/min. This process yielded pure, yellow powders identified as compound 1 (7.5 mg) [19], 2 (4.3 mg) [20], and 3 (6.4 mg) [21].

5.4. Anti-HCV assay

A serial dilution of extract/fraction/isolates (500, 100, 50, 10, and 1 µg/mL) were pre-mixed with HCV and inoculated onto Huh7.5 cells (multiplicity of infection/MOI = 0.5). After 2 hours, the cells were washed with serum-free Dulbecco's modified eagle medium (DMEM). The medium containing the same concentrations of extract/fraction/isolates were added and incubated for 46 hrs. The culture supernatants were collected and the titration of virus infectivity was determined by IFA [14]. The percentage inhibition of samples was compared with the control and the IC_{50} value was analyzed using SPSS probit analysis.

5.5. WST-1 assay

WST-1 assay was performed to analyze the cytotoxicity effect of the compounds on the cells. Huh 7.5 cells were cultured in 96-well plates for 24 hours before drug treatment. A serial dilution of extract/fraction/isolates was prepared and added onto Huh 7.5 cells. After 48 hours, 10 µL of WST-1 reagent (Roche, Mannheim, Germany) was added to each well and the cells were incubated for a further 4 hours. The cell viability was measured using a microplate reader at 450 nm. The viability of drug-treated cells was calculated as a percentage of the untreated control and the CC_{50} was determined by SPSS probit analysis.

5.6. Mode-of-Action of the active compound isolated from *Melicope latifolia*

The mode-of-action experiment was performed to determine the inhibitory stage of compound(s) in HCV life cycle. Three sets of experiments were carried out in parallel to examine the effect of the compounds on viral entry, post-entry and both steps (entry and post entry). Entry step inhibition was determined by adding the test compounds 2 hours during HCV inoculation and incubating with DMEM for 46 hrs. Post-entry step inhibition was analysed by adding the test compounds for 46 hrs after virus infection. Whole step inhibition was determined by adding the test compounds at both 2 hours and 46 hours after HCV inoculation.

5.7. Immunofluorescence analysis (IFA)

Culture supernatants were inoculated in Huh 7.5 cells, which were seeded on glass coverslips in a 24 well plate. After 24 hours of incubation, the cells were fixed with 3.7% paraformaldehyde in Phosphate-buffered saline (PBS) for 10 min and permeabilized in PBS containing 0.1% Triton X-100 for 10 minutes. The human serum antibody was incubated for 1 hour, followed by (FITC-conjugated goat anti-human IgG) (MBL) as the secondary antibody. The cells were washed with PBS, counterstained with Hoechst 33342 solution (Molecular Probes) at room temperature for 10 min, mounted on glass slides, and infected cells were examined under a fluorescence microscope.

5.8. Docking Analysis

Interaction of the compound was predicted by docking analysis with Molegro Virtual Docker ver 5.5 program. Several proteins from the Protein Data Base (www.rcsb.org) were evaluated for their interaction with HCV protein. The docking test begins with ligand preparation by creating 2-D and 3-D structures using the ChemBioOffice Ultra 20.0 program and minimizing energy with MMFF9. Prior to docking the compound, a method validation process was carried out by determining the RMSD value < 2Å. Furthermore, docking was performed on the test compounds to obtain Rerank Scores and ligand-amino acid interactions.

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