SYNTHESIS AND ANTI-HEPATITIS C VIRUS ACTIVITY OF GALLIC ACID DERIVATIVES WITH CHIRAL CENTER

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INTRODUCTION

Hepatitis C is an infectious disease caused by hepatitis C virus (HCV) that attacks the liver, causing inflammation to damage the liver cells. Hepatitis C has become a global health issue since the World Health Organization estimated that about 170 million of the world’s population is currently suffering in hepatitis C. The prevalence of HCV infection varies worldwide. Endemic areas are quite high among Southeast Asia, including in Indonesia, with 7 million people are infected by hepatitis C [1-3]. Various therapies have been applied for the treatment of HCV infections, including the use of combination therapy with interferon alpha or pegylated interferon as an immunomodulator, with Ribavirin, boceprevir or telaprevir as an antiviral nucleoside analog, which is effective to inhibit the growth of cells of hepatitis C [4-7]. Polamreddy et al. reported that until 2011, the standard treatment for hepatitis C was with a combination of pegylated interferon and Ribavirin which is given for period from 24 to 48 weeks. However, this treatment showed severe side effects and effective in only 50-60% of patients [8]. Besides that, recent research revealed that current antiviral treatment of hepatitis C therapy has poor tolerance to some patients and has a high resistance level [6,7]. This fact indicating the search for novel antiviral agents of hepatitis C which are safer and more effective are needed. Gallic acid (1) is a naturally bioactive compound that showed antimicrobial and anti-HSV-2 activity [9,10]. Previous researchers reported that gallic acid (1) containing in ethanolic extract of Chinese herbal Saxifraga melanocentra, showed anti-HCV activity against NS3 serine protease [11,12]. Sharaf et al. in 2012 reported that the gallic acid which is the main component of grape seed extract of Vitis vinifera L. showed the inhibitory effect on cell growth of HCV human hepatoma HepG2 [13]. Simlar with this finding, in 2014, Aoki et al. revealed that gallic acid which was isolated from Kalanchoe pinnata, a member of Crassulaceae family found in tropical areas, showed a strong anti-HCV activity [1-4]. Furthermore, Zuo et al. reported that ester of gallic acid and D-glucose (gallated-D-glucose ester) which has several chiral centers showed the anti-HCV activity 10 times more powerful than gallic acid or alkyl esters gallate which have no chiral center [11]. These previous results indicate that gallic acid derivative with chiral center is potential to be developed as antiviral agent of hepatitis C. Thus, in this work, we aim to synthesize gallic acid derivatives with chiral center, and to evaluate the anti-HCV activity of the derivatives against HCV.

OBJECTIVE

In this work, we aim to synthesize gallic acid derivatives with chiral center and to evaluate its anti-hepatitis C virus (anti-HCV) activity.

METHODS

The target derivatives was started from esterification of commercially available boc deprotection (Boc-L-threonine with allyl bromide, followed by Boc with HCl/EtOAc, amidation, and Sharpless asymmetric dihydroxylation with (DHQ)_2PHAL or (DHQD)_2PHAL as a ligand to give desired gallic acid derivatives. The synthesized gallic acid derivatives were then evaluated for anti-HCV activity and cytotoxicity.

RESULTS

The target derivatives were successfully synthesized in ranging from 20% to 30% of yield. Anti-HCV activity of the derivatives is greatly improved by the presence of chiral center, hydroxyl group, and monomethoxy group on the aromatic ring, with showed no cytotoxicity. This fact revealed that the chiral center, hydroxyl group, and monomethoxy group on the aromatic ring of gallic acid derivatives are responsible for its anti-HCV activity. Moreover, gallic acid derivative which possesses a chiral center of bottom facial stereochemistry was found to have a stronger anti-HCV activity than gallic acid derivative with chiral center of top facial stereochemistry. Suggesting that, bottom facial stereocenter in gallic acid derivative was more effective for anti-HCV activity than the top facial stereocenter.

CONCLUSION

Gallic acid derivatives with chiral center exhibited a greater antiviral activity against HCV than gallic acid. Thus, the derivatives should be considered as a promising candidate for the treatment of HCV infection.

Keywords: Synthesis, Gallic acid derivative, Chiral center, Anti-hepatitis C virus.

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INTRODUCTION

Hepatitis C is an infectious disease caused by hepatitis C virus (HCV) that attacks the liver, causing inflammation to damage the liver cells. Hepatitis C has become a global health issue since the World Health Organization estimated that about 170 million of the world’s population is currently suffering in hepatitis C. The prevalence of HCV infection varies worldwide. Endemic areas are quite high among Southeast Asia, including in Indonesia, with 7 million people are infected by hepatitis C [1-3]. Various therapies have been applied for the treatment of HCV infections, including the use of combination therapy with interferon alpha or pegylated interferon as an immunomodulator, with Ribavirin, boceprevir or telaprevir as an antiviral nucleoside analog, which is effective to inhibit the growth of cells of hepatitis C [4-7]. Polamreddy et al. reported that until 2011, the standard treatment for hepatitis C was with a combination of pegylated interferon and Ribavirin which is given for period from 24 to 48 weeks. However, this treatment showed severe side effects and effective in only 50-60% of patients [8]. Besides that, recent research revealed that current antiviral treatment of hepatitis C therapy has poor tolerance to some patients and has a high resistance level [6,7]. This fact indicating the search for novel antiviral agents of hepatitis C which are safer and more effective are needed. Gallic acid (1) is a naturally bioactive compound that showed antimicrobial and anti-HSV-2 activity [9,10]. Previous researchers reported that gallic acid (1) containing in ethanolic extract of Chinese herbal Saxifraga melanocentra, showed anti-HCV activity against NS3 serine protease [11,12]. Sharaf et al. in 2012 reported that the gallic acid which is the main component of grape seed extract of Vitis vinifera L. showed the inhibitory effect on cell growth of HCV human hepatoma HepG2 [13]. Simlar with this finding, in 2014, Aoki et al. revealed that gallic acid which was isolated from Kalanchoe pinnata, a member of Crassulaceae family found in tropical areas, showed a strong anti-HCV activity [1-4]. Furthermore, Zuo et al. reported that ester of gallic acid and D-glucose (gallated-D-glucose ester) which has several chiral centers showed the anti-HCV activity 10 times more powerful than gallic acid or alkyl esters gallate which have no chiral center [11]. These previous results indicate that gallic acid derivative with chiral center is potential to be developed as antiviral agent of hepatitis C. Thus, in this work, we aim to synthesize gallic acid derivatives with chiral center, and to evaluate the anti-HCV activity of the derivatives against HCV.

The chemical structure of gallic acid (1) and its desired derivatives (compounds 2-5) are displayed in Fig. 1. The synthesis was designed by modifying the carboxyl group of gallic acid with an open-chain moiety of L-threonine-allyl esters, as well as to modify hydroxyl group on the aromatic ring of gallic acid with monomethoxy group in derivative 2 and 3, and with dimethoxy group in derivative 4 and 5. To learn whether the stereochemistry affects antiviral activity, we introduce chiral center with bottom facial stereochemistry in
Fig. 1: Structure of gallic acid 1, target derivatives 2, 3, 4, and 5

derivative 2 and 4, in contrast, with top facial stereochimistry in derivative 3 and 5. Introduction of a chiral center on the target
derivatives is expected to increase the activity and effectiveness of the
derivatives as antiviral agents of hepatitis C.

METHODS

In this research, we synthesized some derivative compounds of gallic
acid with chiral center and examined its anti-HCV activity, cytotoxicity,
and selectivity index (SI). Synthesis of the target derivatives was
started from condensation of commercially available boc deprotection
(Boc)-L-threonine with allyl bromide, followed by Boc with HCl/EDAc
and amidation reaction. Sharpless asymmetric dihydroxylation (DHQ)
with OsO4 in the presence of (DHQ)PHAL or (DHQD)PHAL ligand
to control the stereoselectivity to give desired gallic acid derivatives
with chiral center [15,16]. The synthesis procedures of allyl ester 11
and ester 8 have been described in our previous work [17]. H-nuclear
magnetic resonance (NMR) and 13C-NMR spectra were recorded on
500 MHz JEOL JNM-ECP500 spectrometers using tetramethylsilane
(δ 0), CDCl3 (δ 7.26), DMSO (δ 2.49), or acetone (δ 2.05) as an internal
standard. Mass spectra were recorded on Shimadzu GCMS QP-5000 or
JEOL JMS-AX 700 spectrometers.

Synthesis of amide 9

WSCD.HCl (117 mg, 0.61 mmol) and NMM (0.33 ml, 3.06 mmol) were
added to a mixture solution of L-threonine-allyl-ester ammonium
chloride 8 (100 mg, 0.51 mmol) monomethoxybenzoic acid 6 (130 mg,
0.61 mmol), and HOBt (104 mg, 0.77 mmol) in DMF (5 mL). The
mixture was stirred at rt for 24 hrs. The mixture was then diluted by
addition of EtOAc (50 mL) and washed repeatedly by water (4×25 mL),
and saturated NaCl (2×25 mL). EtOAc phase was dried over MgSO4
anhydrous and evaporated. The residue was flash chromatographed
on silica gel (gradient elution 40:1 to 30:1, CHCl3:MeOH), gave amide
9 (96.1 mg, 58%) as yellow oil.

To a solution of amide 9 (50 mg, 0.12 mmol), (DHQ)PHAL (19 mg,
20 mol%) and NMO (72 mg, 0.66 mmol) in t-BuOH: THF:H2O (4:4:0.8)
was added OsO4 (6 mg, 20 mol%). The resulting mixture was
stirred at rt and monitoring by thin-layer chromatography (TLC) until
disappearance of starting material 9 (1 hr). The reaction was quenched
with the addition of NaSO3 (153 mg) and water (2 mL). The resulting
mixture was extracted by CH2Cl2 (3×15 mL). The combined CH2Cl2
layers were dried over NaSO4 filtered and concentrated under reduced
pressure. The crude residue was purified by column chromatography
on silica gel (gradient elution 40:1 to 8:1, CHCl3:CH2OH) gave a pale
yellow oil of derivative 2 (29.8 mg, 30%). Rf =0.50 (5:1 CHCl3:CH2OH).

H-NMR (500 MHz, acetone-d6): δ 8.30 (1H), 5.19 (dd, J=8.6 Hz and 4.3 Hz, 1H), 4.71 (dd, J=5.2 Hz and 2.3 Hz, 1H), 6.00-5.89 (m, 1H), 5.37 (dd, J=15 Hz and 7.7 Hz, 1H), 5.20 (dd, J=8.4 Hz and 4.2 Hz, 1H), 4.64-4.48 (m, 1H), 4.45-4.40 (m, 1H), 4.32-4.11 (m, 3H), 3.90 (s, 3H), 3.82 (s, 3H), 7.48 (d, J=8.6 Hz, 1H), 7.13 (s, 2H), 4.72-4.66 (m, 1H), 4.42-4.36 (m, 1H), 4.27-4.09 (m, 3H), 3.80 (s, 3H), 3.72-3.63 (m, 1H), 3.56-3.52 (m, 2H), and 1.26 (d, J=6.3 Hz, 3H). 13C-NMR (125 MHz, acetone-d6): δ 171.1, 167.4, 151.2, 138.9, 133.2, 130.7, 117.9, 107.6, 68.2, 65.9, 60.6, 59.1, and 20.7 HRMS ESI-calcd for C16H14NO3 [M+H]+: 292.1017, found: 292.0954.

Synthesis of derivative 4

To a solution of amide 9 (50 mg, 0.12 mmol), (DHQD)PHAL (19 mg,
20 mol%) and NMO (72 mg, 0.66 mmol) in t-BuOH: THF:H2O (4:4:0.8)
was added OsO4 (6 mg, 20 mol%). The resulting mixture was
stirred at rt and monitoring by thin-layer chromatography (TLC) until
disappearance of starting material 9 (1 hr). The reaction was quenched
with the addition of NaSO3 (80 mg) and water (2 mL). The resulting
mixture was extracted by CH2Cl2 (3×15 mL). The combined CH2Cl2
layers were dried over Na2SO4 filtered and concentrated under reduced
pressure. The crude residue was purified by column chromatography
on silica gel (gradient elution 16:1 to 1:1, CHCl3:CH2OH) gave a colorless oil of derivative 4 (32.1 mg, 20%). Rf = 0.46 (5:1 CHCl3:CH2OH).

H-NMR (500 MHz, acetone-d6): δ 8.30 (1H), 5.19 (dd, J=8.6 Hz and 4.3 Hz, 1H), 4.71 (dd, J=5.2 Hz and 2.3 Hz, 1H), 6.00-5.89 (m, 1H), 5.37 (dd, J=15 Hz and 7.7 Hz, 1H), 5.20 (dd, J=8.4 Hz and 4.2 Hz, 1H), 4.64-4.48 (m, 1H), 4.45-4.40 (m, 1H), 4.32-4.11 (m, 3H), 3.90 (s, 3H), 3.82 (s, 3H), 7.48 (d, J=8.6 Hz, 1H), 7.13 (s, 2H), 4.72-4.66 (m, 1H), 4.42-4.36 (m, 1H), 4.27-4.09 (m, 3H), 3.80 (s, 3H), 3.72-3.63 (m, 1H), 3.56-3.52 (m, 2H), and 1.26 (d, J=6.3 Hz, 3H). 13C-NMR (125 MHz, acetone-d6): δ 171.1, 167.4, 151.2, 138.9, 133.2, 130.7, 117.9, 107.6, 68.2, 65.9, 60.6, 59.1, and 20.7 HRMS ESI-calcd for C16H14NO3 [M+H]+: 292.1017, found: 292.0954.

Synthesis of derivative 3

To a solution of amide 9 (50 mg, 0.12 mmol), (DHQ)PHAL (19 mg,
20 mol%) and NMO (72 mg, 0.66 mmol) in t-BuOH: THF:H2O (4:4:0.8)
was added OsO4 (6 mg, 20 mol%). The resulting mixture was
stirred at rt and monitoring by thin-layer chromatography (TLC) until
disappearance of starting material 9 (1 hr). The reaction was quenched
with the addition of NaSO3 (80 mg) and water (2 mL). The resulting
mixture was extracted by CH2Cl2 (3×15 mL). The combined CH2Cl2
layers were dried over Na2SO4 filtered and concentrated under reduced
pressure. The crude residue was purified by column chromatography
on silica gel (gradient elution 16:1 to 1:1, CHCl3:CH2OH) gave a colorless oil of derivative 3 (121 mg, 20%). Rf = 0.46 (5:1 CHCl3:CH2OH).

H-NMR (500 MHz, acetone-d6): δ 8.30 (1H), 5.19 (dd, J=8.6 Hz and 4.3 Hz, 1H), 4.71 (dd, J=5.2 Hz and 2.3 Hz, 1H), 6.00-5.89 (m, 1H), 5.37 (dd, J=15 Hz and 7.7 Hz, 1H), 5.20 (dd, J=8.4 Hz and 4.2 Hz, 1H), 4.64-4.48 (m, 1H), 4.45-4.40 (m, 1H), 4.32-4.11 (m, 3H), 3.90 (s, 3H), 3.72-3.63 (m, 1H), 3.56-3.52 (m, 2H), and 1.26 (d, J=6.3 Hz, 3H). 13C-NMR (125 MHz, acetone-d6): δ 171.1, 167.4, 151.2, 138.9, 133.2, 130.7, 117.9, 107.6, 68.2, 65.9, 60.6, 59.1, and 20.7 HRMS ESI-calcd for C16H14NO3 [M+H]+: 292.1017, found: 292.0954.
Synthesis of derivative 5
To a solution of amide 10 (50 mg, 0.11 mmol), (DHQD) PHAL (18 mg, 20 mol%), and NMO (69 mg, 0.55 mmol) in t-BuOH: THF:H₂O (2:2:0.4) was added OsO₄ (6 mg, 20 mol%). The resulting mixture was stirred at rt and monitoring by TLC until disappearance of starting material 10 (1 hr). The reaction was quenched with the addition of Na₂SO₄ (80 mg) and water (2 mL). The resulting mixture was extracted with CH₂Cl₂ (3×15 mL). The combined CH₂Cl₂ layers were dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude residue was purified by column chromatography on silica (gradient elution 35:1 to 1:1, CHCl₃:CH₂OH) gave a colorless oil of derivative 5 (8.2 mg, 20%). Rf = 0.19 (5:1 CHCl₃:CH₂OH). ¹H-NMR (500 MHz, acetone-d₆): δ 7.49 (d, J = 8.6 Hz, 1H), 7.20 (s, 2H), 4.72-4.66 (m, 1H), 4.45-4.40 (m, 1H), 4.32-4.11 (m, 3H), 3.90 (s, 3H), 3.81 (s, 3H), and 1.24 (d, J = 6.3 Hz, 3H). ¹³C-NMR (125 MHz, acetone-d₆): δ 171.5, 167.6, 153.9, 145.3, 140.4, 133.7, 130.7, 108.9, 70.6, 67.0, 60.7, 59.5, 56.3, 56.3, and 28.2. and 20.4. HRMS ESI-calcd for C₁₄H₁₄NO₅ [M+H]⁺: 374.1451, found: 374.1453.

Anti-HCV activity assay
Huh7it-1 cells were seeded into 48-well plates at 5.0×10⁴ cells/well 1 day before virus infection. Two-fold serial dilutions of gallic acid derivatives were prepared in culture medium in 96-well plates. The same volume of HCV (5.0×10⁴ focus-forming unit) was mixed with the samples and immediately inoculated into the Huh7it-1 cells. After incubation for 2 hrs at 37°C, the cells were rinsed twice with serum-free medium to remove the residual virus and incubated for an additional 46 hrs with the same medium. At 48 hrs after post infection, the culture supernatants were collected and used for virus titration.

Cytotoxicity assay
Cytotoxicity of the pure synthesized gallic acid derivatives against Huh7it-1 cells was determined using MTT assay (Sigma-Aldrich). Cells (2.0×10⁴ cells/well) were seeded into 96-well plates and treated with various concentrations of the synthesized derivatives for 48 hrs. The medium was replaced with 100 µl of MTT-containing medium and incubated for an additional 4 hrs. The medium was removed, and 100 µl of DMSO was added to dissolve the generated precipitates. Absorbance at 550 nm was measured using a GhMax-Multi Microplate Multimode Reader (Promega). The percentage of viable cells was plotted versus the concentration of the test compound. The concentration by which to mediate 50% cytotoxicity (CC₅₀) was determined by linear regression analysis.

RESULTS AND DISCUSSIONS
Synthesis of the desired derivatives (2-5)
Synthesis of the target derivatives was started from esterification of Boc-L-threonine (12) with allyl bromide to give allyl ester (11). Boc of 11 with HCl/EtOAc afforded ester 8. In the next pathway, there are two pathways. In the first pathway, amidation of 8 with monomethoxy benzoic acid 6 to give amide 9. In the second pathway, amidation of 8 with dimethoxy benzoic acid 7, produced amide 10. Subsequently, Sharpless asymmetric DHQ of 9 with OsO₄ and (DHQD) PHAL ligand to give hydroxylated amide products as a 10:1 diastereomeric ratio, which can be separated by flash column chromatography to give pure derivative 2 in 30% of yield. Sharpless asymmetric DHQ of 9 with OsO₄ and (DHQD) PHAL followed by purification on flash column chromatography to produce pure derivative 3 in 25% of yield. Whereas, Sharpless asymmetric DHQ of 9 with (DHQ) PHAL and (DHQD) PHAL ligand followed by purification on flash column chromatography to produce pure derivative 5 in 25% yield and 20% yield, respectively. Monomethoxy benzoic acid 6 and dimethoxy benzoic acid 7 were prepared from starting material gallic acid by methylation with Mel/K₂CO₃ and hydrolysis with LiOH. Synthesis pathway of target derivatives 2-5 is displayed in Scheme 1.

Antiviral activity and cytotoxicity
After completion of the synthesis, antiviral activity of gallic acid, target derivative 2, 3, 4, and 5 were evaluated against HCV strain JFH1, antiviral activity represented by IC₅₀. The smaller IC₅₀ value, the higher antiviral activity. Toxicities of gallic acid and the derivatives against Huh 7it-1 cells were determined MTT cell proliferation assay and represented by CC₅₀, the higher CC₅₀ the lower cytotoxicity. Whereas, SI showed the selectivity of the test compound to inhibit the HCV infection with did not show any toxicity on the Huh-7it-1 cell (human hepatocarcinoma 7 it-1 cells). The higher SI value, more selective compound to inhibit HCV infection. Antiviral activity and cytotoxicity of gallic acid, target derivative 2, 3, 4, and 5 are summarized in Table 1.

As shown in Table 1, gallic acid, derivative 2 and 3 have CC₅₀ value over than 100 µg/mL which are assigned as nontoxic compounds, whereas derivative 4 and 5 with a CC₅₀ value of 18.3 and 27.2 µg/mL, respectively, are assigned as toxic compounds. This result is suggesting that replacing two hydroxyl groups of gallic acid with methoxy groups will increase the cytotoxicity. Compared to gallic acid (1), antiviral activity and SI of derivative 2 and 3 are greatly improved by the presence of chiral center, hydroxyl group, and monomethoxy group on the aromatic ring. This fact suggested that the chiral center, hydroxyl group, and monomethoxy group on the aromatic ring are very important for selectivity and anti-HCV activity. Furthermore, derivative 2 which contains hydroxyl group with bottom facial stereochemistry on the chiral center, showed higher selectivity and stronger anti-HCV activity compared to derivative 3 which has hydroxyl group with top facial stereochemistry on the chiral center, suggesting that bottom facial stereocenter in derivative 2 was more effective for anti-HCV activity than top facial stereocenter in derivative 3.

Scheme 1: Synthesis pathway of target derivative 2, 3, 4, and 5
**CONCLUSION**

Gallic acid derivatives with chiral center have been synthesized. The target derivatives exhibited a greater antiviral activity against HCV than gallic acid. Thus, the derivatives of gallic acid with chiral center should be considered as a promising candidate for the treatment of HCV infection.

**ACKNOWLEDGMENTS**

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**REFERENCES**


**Table 1: Anti-HCV activity and cytotoxicity of gallic acid (1), target derivative 2, 3, 4, and 5**

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<th>S. No</th>
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HCV: Hepatitis C virus, SI: Selectivity index