



Synthesis and Cytotoxic Activities of Hexyl-esters Derivatives of Gallic Acid Against MCF-7 Cell Line

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ABSTRACT

Gallic acid is found in many plants, fruits, and foods where the anti-cancer activity is found. However, gallic acid has a problem on the high polarity and low bio availability. So, it takes molecular modifications in order to increase its lipophilicity, which is expected to increase bio availability and cytotoxic activity of gallic acid. Hexyl esters derivatives of gallic acid were synthesized and characterized by spectrometer ¹H-NMR, ¹³C-NMR, mass spectrometry and infrared spectrophotometer (FTIR). All compounds were then evaluated for cytotoxic activity on MCF-7 cell line using MTT method. Compound cis-2'-hexenyl-3,4,5-trimethoxygallate (19) had the lowest IC₅₀ value compared with gallic acid and other derivatives hexyl esters. IC₅₀ value of cis-2'-hexenyl-3,4,5-trimethoxygallate (19) is 14.48 µg/ml. Compound (19) also has approached with IC₅₀ values of gossypol as a positive control. Compound (19) is a potential compound to inhibit growth of MCF-7 cell line.

Keywords: Gallic acid, MCF-7, Cytotoxicity assay, Synthesis of hexyl esters gallate derivative.

INTRODUCTION

Breast cancer is the second most common cancer in the world and the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). Breast cancer ranks as the fifth cause of death from cancer overall¹. A lot of research to

synthesis new potential anticancer drugs. Although many potential anticancer drugs have been made but the medical need is still largely unmet due to many factors among which the lack of selectivity of conventional drugs leading to toxicity, the metastatic spreading, and multi-drug resistance; (MDR). Therefore, the search for novel and selective anticancer agents is highly required due to currently available anticancer drugs problems.^{2,3}



In recent decades, the development of novel and advanced cancer therapies common use natural plants, fruits, or foods as a valuable resource. Gallic acid (3,4,5-trihydroxybenzoic acid) is compound which is widely distributed in various plants, fruits, and foods. Gallic acid was demonstrated to have various biological activities including antibacterial, antiviral, and anti-inflammatory, in which the antitumor activity is most striking.³ Growth of human breast cancer cell, MCF-7, is significantly reduced by treatment with gallic acid. Gallic acid causes apoptosis induction and cells treated with gallic acid showed significantly downregulated of Bcl-xL protein and upregulation of Bak and Bad proteins.⁴

In previous study, we were done with in silico study about hexyl esters derivatives of gallic

acid. Hexyl esters of trimethoxygallic acid showed more interaction of hydrophobic amino acid than gallic acid.⁵ This leads to increased bond stability with the receptor. Compound *cis*-2'-hexenyl-3,4,5-trimethoxygallic acid serve as potential inhibitor of anti-apoptotic Bcl-xL protein because had the lowest ΔG dan K_i . There is also pi-pi interaction with TYR105 which adds stability to protein-inhibitor interactions and protein complexes.⁶

To prove the docking result, we had synthesized the hexyl esters derivative of gallic acid (Fig. 1) and further testing the cytotoxic activity against MCF-7 cell line. The in vitro result then compared to in silico docking result to get a potent anticancer drug for breast cancer.

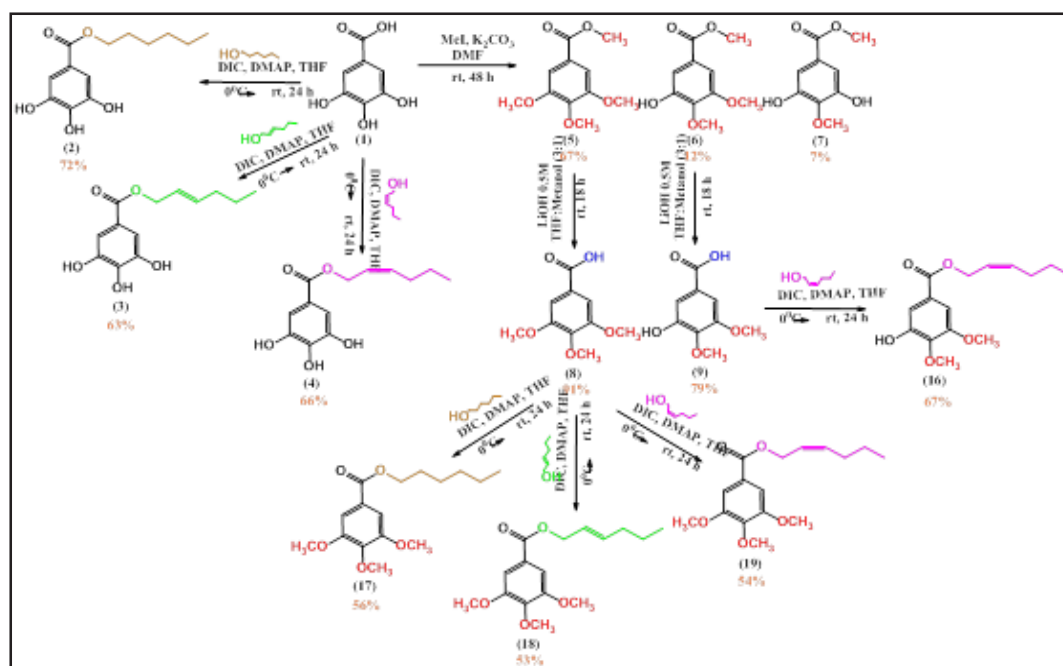


Fig. 1. Scheme of synthesis of gallic acid derivative

EXPERIMENTAL

Synthesis reaction

Chemicals, reagents and solvents were purchased from Merck & Co. and Sigma-Aldrich. ¹H-NMR (proton nuclear magnetic resonance) was measured using JEOL JNM-ECP (500 Hz) in Acetone-*d*₆ as solvent. ¹³C-NMR (carbon nuclear

magnetic resonance) was measured using JEOL JNM-ECP (125 Hz) in Acetone-*d*₆ as solvent. The chemical shifts were reported in ppm and coupling constants were measured in Hertz (Hz). Reactions were monitored with thin layer chromatography using aluminum sheets silica gel 60 F₂₅₄ 2x5 cm (Merck & Co.). Ultra-violet light (254 nm) and iodine vapour were used to visualize the chromatograms.

Mass spectra (MS) were recorded on Shimadzu GCMS QP-5000, IR spectrum were recorded using IR (JASCO FT/IR-420 spectrophotometer).

Methylation reaction

A solution of gallic acid (17.65 mmol), and K_2CO_3 (52.83 mmol) in DMF (25 mL) were added Methyl iodide (52.83 mmol) and stirred at room temperature for 48 hours. The reaction mixture was then added 75 ml ethyl acetate and then washed with 3x25 ml saturated $NaHCO_3$ and 2x25 ml saturated NaCl, sequently. The organic phase was then added Mg_2SO_4 anhydrate to take out the water residue. The filtrate was then drying to obtain the crude product. The product was purified using chromatography column with chloroform as mobile phase and give 3 products : 4-monomethoxymethyl gallate, 4,5-dimethoxymethyl gallate, 3,4,5-trimethoxymethyl gallate.

Hidrolisis reaction

A solution of each methylation product and LiOH monohydrate 0.5 M in mixture THF:Methanol (3:1) were stirred at room temperature for 24 hours. The reaction mixture was then acidified using $KHSO_4$ 1M until pH-3, then added 15 mL of aquadest, and extracted with 3x15 ml ethyl acetate. The organic phase was washed with saturated NaCl and then added Mg_2SO_4 anhydrate to take out the water residue. The filtrate was then drying to obtain the product and give 3 products: 4-monomethoxygallic acid, 4,5-dimethoxygallic acid, 3,4,5-trimethoxygallic acid, respectively.

Esterification reaction

A solution of gallic acid or 3,4,5-trimethoxygallic acid (1 mmol), alcohols (2 mmol) and 1.5 mmol diisopropyl carbadiimide (DIC) as catalisator in tetrahydrofurane (10 mL) were stirred in 0 °C for 30 min. then added solution of 0,1 mmol N,N-Dimethylaminopiridine (DMAP) in 1 ml THF. This mixture was stirred at room temperature for 24 hours. The reaction mixture was then added aquadest and extracted with chloroform. The organic phase was then added Mg_2SO_4 anhydrate to take out the water residue. The filtrate was then drying to obtain the crude product. The product was purified using chromatography column with appropriate mobile phase.

Cytotoxicity effects

The inhibitory effect of synthesized compounds against colorectal adenocarcinoma cell line, HT-29, was determined using MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. First, cells were seeded in 96-well plates at 5000 cells/well and allowed to attach in 24 h Media was renewed and the cells were added with several concentrations of the extract (3.125–100 ppm) incubated for an additional 24 h. After 24 h, 20 μ M of MTT solution (0.5 mg/ml MTT solution in media) was added to each well and incubated for 4 h at 37 °C. The supernatant was aspirated and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 100 μ l of dimethyl sulphoxide (DMSO). Finally, the absorbance was monitored by a microplate reader at a wavelength of 570 nm. The percentage of viable cells was plotted versus the concentration of the test compound. The concentration by which to mediate 50% cytotoxicity (IC_{50}) was determined by linear regression analysis.

RESULT AND DISCUSSION

Synthesis of the desired compound

The compounds were synthesized as per Fig. 1 and the structure was elucidated by spectroscopic data, i.e IR, 1H -NMR, ^{13}C -NMR and LCMS are presented as follows:

Hexylgallate (2)

IR (KBr): ν cm^{-1} , 3326.61 (OH), 2927.41 (Al. C–H), 1685.48 (C=O), 1535.06 (Ar C=C), 1222.65 (C–O); 1H NMR (Acetone- d_6) δ : 7.11 (s, 2H, Ar-CH), 4.19 (t, 2H, OCH_2CH_2), 1.74 – 1.66 (m, 2H, $CH_2CH_2CH_2$), 1.47 – 1.39 (m, 2H, $CH_2CH_2CH_2$), 1.36 – 1.30 (m, 4H, $CH_2CH_2CH_2$), 0.89 (t, 3H, CH_2CH_3). ^{13}C NMR (Acetone- d_6) δ : 166.72, 146.02 (2C), 138.48, 122.03 (2C), 109.64, 64.91, 42.04, 32.18, 26.41, 23.59, 14.24. LCMS m/z : $[M^+]$ 254.1154 ($C_{13}H_{18}O_5$).

Trans-2'-hexenylgallate (3)

IR (KBr): ν cm^{-1} , 3272.61 (OH), 2927.41 (Al. C–H), 1685.48 (C=O), 1535.06 (Ar C=C), 1226.5 (C–O); 1H NMR (Acetone- d_6) δ : 7.08 (s, 2H, Ar-CH), 5.84 – 5.76 (m, 1H, $CH_2CH=CH_2$), 5.67 – 5.59 (m, 1H, $CH=CHCH_2$), 4.61 (dd, 2H, OCH_2CH_2), 1.41 – 1.31 (q, 2H, $CH_2CH_2CH_2$), 1.10 – 1.02 (s, 2H,

CH₂CH₂CH₃) 0.86 (t, 3H, CH₂CH₃). ¹³C NMR (Acetone-d₆) d: 166.50, 146.08 (2C), 136.03, 131.22, 125.56 (2C), 109.66 (2C), 65.46, 42.18, 23.53, 13.82. LCMS m/z: [M⁺] 252.0998 (C₁₃H₁₆O₅).

Cis-2'-hexenylgallate (4)

IR (KBr): ν cm⁻¹, 3326.61 (OH), 2927.41 (Al. C-H), 1685.48 (C=O), 1535.06 (Ar C=C), 1222.65 (C-O); ¹H NMR (Acetone-d₆) d: 7.01 (s, 2H, Ar-CH), 5.65 – 5.54 (m, 2H, CH₂CH=CH), 4.72 (d, 2H, OCH₂CH₂), 2.14 – 2.06 (q, 2H, CH₂CH₂CH₂), 1.42 – 1.32 (m, 2H, CH₂CH₂CH₃) 0.92 (t, 3H, CH₂CH₃). ¹³C NMR (Acetone-d₆) d: 166.52, 157.91, 146.77 (2C), 135.11, 125.14 (2C), 109.59 (2C), 60.37, 23.13, 21.43, 13.85. LCMS m/z: [M⁺] 252.0997 (C₁₃H₁₆O₅).

Cis-2'-hexenyl-3,4-dimethoxygallate (16)

IR (KBr): ν cm⁻¹, 3403.74 (OH), 2956.34 (Al. C-H), 1683.35 (C=O), 1587.13 (Ar C=C), 1324.86 (C-O); ¹H NMR (Acetone-d₆) d: 7.20 (s, 1H, Ar-CH), 7.16 (s, 1H, Ar-CH), 5.73 – 5.64 (m, 2H, CH₂CH=CH), 4.83 (d, 2H, OCH₂CH₂), 3.89 (s, 3H, OCH₃), 3.83 (s, 3H, OCH₃), 2.20 – 2.15 (q, 2H, CH₂CH₂CH₂), 1.47 – 1.39 (s, 2H, CH₂CH₂CH₃) 0.93 (t, 3H, CH₂CH₃). ¹³C NMR (Acetone-d₆) d: 166.10, 163.15, 153.75, 135.56, 132.24, 126.31, 124.68,

105.49, 60.96, 60.59, 58.21, 29.65, 23.06, 13.72. LCMS m/z: [M⁺] 280.1311 (C₁₅H₂₀O₅).

Hexyl-3,4,5-trimethoxygallate (17)

IR (KBr): ν cm⁻¹, 2935.13 (Al. C-H), 1714.41 (C=O), 1589.06 (Ar C=C), 1224.58 (C-O); ¹H NMR (Acetone-d₆) d: 7.30 (s, 2H, Ar-CH), 4.29 (t, 2H, OCH₂CH₂), 3.88 (s, 6H, OCH₃), 3.79 (s, 3H, OCH₃), 1.79 – 1.72 (m, 2H, CH₂CH₂CH₂), 1.49 – 1.42 (m, 2H, CH₂CH₂CH₂), 1.38 – 1.33 (m, 4H, CH₂CH₂CH₂), 0.90 (t, 3H, CH₂CH₃). ¹³C NMR (Acetone-d₆) d: 166.69, 154.45 (2C), 143.63, 126.73, 107.86 (2C), 65.91 60.94, 56.79 (2C), 32.49, 26.71, 23.52 (2), 14.57. LCMS m/z: [M⁺] 296.1624 (C₁₆H₂₄O₅).

Trans-2'-hexenyl-3,4,5-trimethoxygallate (18)

IR (KBr): ν cm⁻¹, 2958.27 (Al. C-H), 1714.41 (C=O), 1589.06 (Ar C=C), 1222.65 (C-O); ¹H NMR (Acetone-d₆) d: 7.24 (s, 2H, Ar-CH), 5.87 – 5.77 (m, 1H, CH₂CH=CH), 5.70 – 5.58 (m, 1H, CH=CHCH₂), 4.69 (dd, 2H, OCH₂CH₂), 3.82 (s, 6H, OCH₃), 3.74 (s, 3H, OCH₃), 2.04 – 2.02 (q, 2H, CH₂CH₂CH₂), 1.42 – 1.32 (m, 2H, CH₂CH₂CH₃) 0.85 (t, 3H, CH₂CH₃). ¹³C NMR (Acetone-d₆) d: 166.47, 154.46 (2C), 143.69, 136.91, 126.59, 125.65, 107.91 (2C), 64.45, 60.94, 56.81 (2C), 35.27, 23.09, 14.17. LCMS m/z: [M⁺] 294.1467 (C₁₆H₂₂O₅).

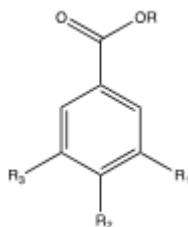


Table. 1 : Physicochemical data of title compounds

Compound	Molecular formula	R	R ₁	R ₂	R ₃	MW	Yield(%)	R _f
2	C ₁₃ H ₁₈ O ₅	-C ₆ H ₁₃	-OH	-OH	-OH	254.11	72	0.70 ^a
3	C ₁₃ H ₁₆ O ₅	-C ₆ H ₁₂	-OH	-OH	-OH	252.10	63	0.65 ^a
4	C ₁₃ H ₁₆ O ₅	-C ₆ H ₁₂	-OH	-OH	-OH	252.10	66	0.64 ^a
16	C ₁₅ H ₂₀ O ₅	-C ₆ H ₁₂	-OCH ₃	-OCH ₃	-OH	280.13	67	0.32 ^b
17	C ₁₆ H ₂₄ O ₅	-C ₆ H ₁₃	-OCH ₃	-OCH ₃	-OCH ₃	296.16	56	0.75 ^b
18	C ₁₆ H ₂₂ O ₅	-C ₆ H ₁₂	-OCH ₃	-OCH ₃	-OCH ₃	294.15	53	0.65 ^b
19	C ₁₆ H ₂₂ O ₅	-C ₆ H ₁₂	-OCH ₃	-OCH ₃	-OCH ₃	294.15	54	0.64 ^b

a Mobile phase : chloroform : methanol (5:1)

b Mobile phase : chloroform

Cis-2'-hexenyl-3,4,5-trimethoxygallate (19)

IR (KBr): ν cm^{-1} , 2958.27 (Al. C–H), 1714.41 (C=O), 1589.06 (Ar C=C), 1220.72 (C–O); ^1H NMR (Acetone- d_6) δ : 7.29 (s, 2H, Ar-CH), 5.73 – 5.61 (m, 2H, $\text{CH}_2\text{CH}=\text{CH}$), 4.85 (d, 2H, OCH_2CH_2), 3.86 (s, 6H, OCH_3), 3.78 (s, 3H, OCH_3), 2.20 – 2.15 (q, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 1.47 – 1.38 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 0.92 (t, 3H, CH_2CH_3). ^{13}C NMR (Acetone- d_6) δ : 166.24, 154.14 (2C), 143.38, 135.77, 126.25, 124.85, 107.60 (2C), 61.29, 60.64, 56.49 (2C), 29.84, 23.24, 13.92. LCMS m/z : [M^+] 294.1465 ($\text{C}_{16}\text{H}_{22}\text{O}_5$).

After completion of the synthesis, cytotoxic activity of gallic acid, target derivative 2, 3, 4, 16, 17, 18, and 19 were determined MTT cell proliferation assay against MCF-7 cell line, cytotoxic activity represented by IC_{50} . The smaller IC_{50} value, the higher cytotoxic activity. From the MTT assay result, compound 19 showed the best cytotoxic activity than other compound, and the IC_{50} value of compound 19 similar to IC_{50} value of Gossypol as positive control. This result was agree with in silico study in previous research by Paramita, 2017. Cytotoxicity assay of gallic acid derivative are summarized in Table. 2 as follow.

Table. 2 : Cytotoxic activity of hexyl esters derivatives of gallic acid

Compound	IC_{50} ($\mu\text{g}/\text{ml}$)
Gallic acid	32.25 ± 0.014
2	24.38 ± 0.014
3	21.28 ± 0.030
4	20.80 ± 0.087
16	18.93 ± 0.062
17	16.54 ± 0.012
18	14.93 ± 0.000
19	14.48 ± 0.087
Gossypol	10.90 ± 0.000

As shown in Table. 2, seven derived compounds had greater inhibitory activity against MCF-7 cell line than gallic acid. Gallic acid has an IC_{50} value of $32.25 \mu\text{g}/\text{ml}$. The results are similar to IC_{50} of gallic acid in previous studies, i.e. $36 \mu\text{g}/\text{ml}$.⁷ Increased lipophilicity of the gallic acid derivative (alkyl ester derivative) will increase the cytotoxic activity. In the previous study, it was shown

that alkyl esters of gallic acid had better cytotoxic effects than gallic acid. This is due to an increase in lipophilicity that contributes in increasing affinity for cell membrane and permeability.⁸ IC_{50} values of hexylgallate 2, trans-2'-hexenylgallate 3, and cis-2'-hexenylgallate 4 are $24.38 \mu\text{g}/\text{ml}$, $21.28 \mu\text{g}/\text{ml}$, $20.80 \mu\text{g}/\text{ml}$, respectively, where the value is lower than the gallic acid.

In the double-bonded hexenyl ester compound, both the cis 4 and trans 3 positions exhibit lower IC_{50} than the hexyl ester compound without the double bond 2. This suggests that the presence of double bonds may increase the cytotoxic activity of the compound. In addition, double bond positions with cis configurations in compounds 4 and 19 exhibit slightly better activity than trans configurations in compounds 3 and 18. In ester compounds without methoxy groups, ie hexylgallate 2, trans-2'-hexenylgallate 3, and cis-2'-hexenylgallate 4, showed less cytotoxic activity than those with methoxy groups. In compounds with the same alkyl ester, cis-2'-hexenyl ester, in compounds 2, 16, 19, with differences in the number of methoxy groups, also shows increased cytotoxic activity as the number of methoxy groups increases. The bonding sites of the Bcl-xL protein are mostly composed of non-polar amino acids.⁹ Thus the methoxy group has a more stable bond on the protein binding site of Bcl-xL compared with the hydroxy group.

From the MTT test results, it is known that the cis-2'-hexenyl-3,4,5-trimethoxygallate 19 compound exhibited the best activity with the lowest IC_{50} value compared to the other gallic acid derivative compounds and the results obtained supported the previous results of in silico study by Paramita *et al.*, 2017. Compound 19 also had an IC_{50} value close to the IC_{50} value of gossypol as a positive control. Compound 19 is a potential compound in inhibiting growth of MCF-7 cell line because has the best in vitro activity between synthesized gallic acid derivatives.

CONCLUSION

We described synthesis of hexyl esters derivatives of gallic acid for *in vivo* cytotoxic activities

against MCF-7 cell line. Among all the compounds synthesized, compounds 19 exhibited most potent cytotoxic activity and had an IC_{50} value close to the IC_{50} value of gossypol. Based on the findings of these *in vitro* results, further studies need to be carried out to investigate *in vivo* assays and toxicological studies.

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