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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Quercetin analogs with high fetal hemoglobin-inducing activity

Wachirachai Pabuprapap[1] · Yanisa Wassanatip[2] · Pichit Khetkam[1] · Waraluck Chaichompoo[1] · Sukanya Kunkaewom[1] · Pongpan Senabud[1] · Janejira Hata[1] · Ratchanaporn Chokchaisiri[3] · Saovaros Svasti[2] · Apichart Suksamrarn[1]

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Abstract

β-Thalassemia is the major health problems in developing countries, when affected patients and healthy carriers are numerous, resulting a total absence or severe decrease in the production of *β*-globin chains. The use of chemical agents for increasing the production of fetal hemoglobin (HbF) by reactivating *γ*-globin gene to balance excess *α*-globin chains is an alternative therapeutic approach. Therefore, the search for molecules exhibiting the property of inducing *γ*-globin gene expression is of great interest. In this report, we discovered that quercetin (**1**), the major flavonoid isolated from the heartwoods of the medicinal plant *Anaxagorea luzonensis* promoted the expression of *γ*-globin gene. Chemical modification of **1** to fourteen methyl ether analogs (**2**−**15**) was conducted. The structures of these compounds were established on the basis of their spectroscopic data and by comparison with those of the reported values. The parent flavonoid and its chemically modified analogs were investigated for their *γ*-globin gene induction for the first time. The parent compound **1** exhibited less induced *γ*-globin gene expression than cisplatin and hemin, the positive controls. 3,4′-Di-*O*-methylquercetin (**7**), the modified analog, significantly enhanced *γ*-globin gene expression with 2.6-fold change at 8 μM, which was slightly higher than cisplatin and hemin. Moreover, compounds **1** and **7** displayed less cytotoxic activity against K562::$\Delta^{G}\gamma^{A}\gamma$EGFP cells than cisplatin. Structure-activity relationship (SAR) study revealed that the methoxyl groups at the 3- and 4′-positions and the free hydroxyl group at the 7-position are required for strong HbF-inducing activity.

Keywords Quercetin · Methylated quercetin · Structure-activity relationship · *β*-Thalassemia · Hemoglobin F

Introduction

β-Thalassemia is one of the major types of thalassemia that is hereditary blood disorders characterized by anomalies in the *β*-globin gene (HBB) on chromosome 11 (Cao and Galanello 2010). It is identified by the absence or decreased production of *β*-globin chain synthesis in human erythroid cells. The continued production of normal levels of the *α*-globin chain exceeds the binding capacity of the *β*-globin chain, leading to globin chain imbalance. Excess *α*-globin chains precipitate in erythroid progenitor cells, resulting in a buildup of reactive oxygen species, causing ineffective erythropoiesis (Liu et al. 2013). The main cores of therapy in *β*-thalassemia patients now are a regular schedule blood transfusion supplemented with iron chelating agents. The only curative treatment is stem cell transplantation. However, it has some limitations such as it is hard to find HLA match donor and high cost (Rund and Rachmilewitz 2005). In recent years, the use of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) inducing drugs is regarded as the appropriate therapy for hemoglobinopathies. HbF expression in *β*-thalassemia will decrease the accumulation and precipitation of *α*-globin chains, and thus reduces the unproductive erythropoiesis (Fard et al. 2013), resulted in better conditions and

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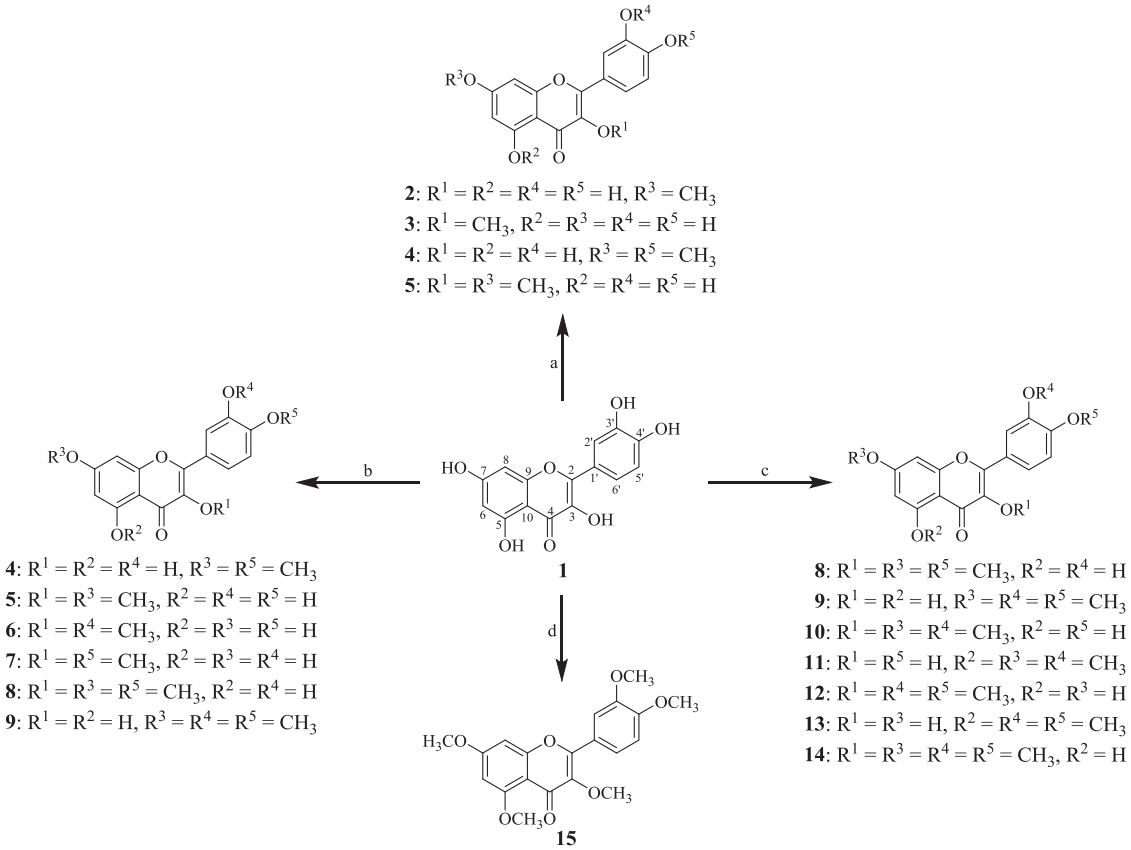
✉ Apichart Suksamrarn
asuksamrarn@yahoo.com
s_apichart@ru.ac.th

[1] Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand

[2] Thalassemia Research Center, Institute of Molecular Biosciences and Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

[3] Department of Chemistry, School of Science, University of Phayao, Phayao 56000, Thailand



Scheme 1 Synthesis of methylated quercetin analogs **2**–**15**. Reagents and conditions: **a** (1) CH_3I (1.0 eqv.), K_2CO_3 (1.25 eqv.), acetone, r.t., 2 h; (2) CH_3I (0.5 eqv.), r.t., 1 h; **b** (1) CH_3I (2.0 eqv.), K_2CO_3 (2.5 eqv.), acetone, r.t., 2 h; (2) CH_3I (1.0 eqv.), r.t., 1 h; **c** (1) CH_3I (3.0 eqv.), K_2CO_3 (3.75 eqv.), acetone, r.t., 2 h; (2) CH_3I (1.5 eqv.), r.t., 1 h; **d** (1) K_2CO_3 (10.0 eqv.), acetone, reflux, 30 min.; (2) CH_3I (excess), reflux, 24 h

transfusion independence of the patients. Accordingly, the stimulation of γ-globin chain production is an alternative treatment for β-thalassemia and sickle cell anemia patients. Although the pharmacological agents for HbF induction, such as 5-azacytidine, cisplatin and hydroxyurea, have been studied for many years, they have low efficiency and high toxicity (El-Beshlawy et al. 2009). Due to these limitations, the research of new types of pharmacological agents that can induce HbF with greater potency and less toxicity seems to be necessary.

Plant-derived natural products have long been and will continue to be extremely important as sources of medicinal agents and models for design, synthesis, and structural modification of novel substances for treating diseases. In many cases, it was found that analogs of compounds derived from structural modification exhibited higher biological activity. However, research on the therapeutic effects of plants on thalassemia has not much been reported. We previously discovered that the diterpenoids from the aerial parts of *Curcuma comosa* enhanced HbF-induction potency using a K562 reporter cell line with the enhanced green fluorescence protein (EGFP) gene under the control of a $^{G}\gamma$-globin promoter (Chokchaisiri et al. 2010). We have also found that the curcuminoids isolated from *Curcuma longa*, particularly the modified analog hexahydrobisdemethoxycurcumin, showed potential γ-globin enhancement (Chaneiam et al. 2013). Quercetin (3,5,7,3′,4′-pentahydroxyflavone, **1**) (Scheme 1) is a ubiquitous bioflavonoid present in most of the plants, fruits, and vegetables (D'Andrea 2015). Previous phytochemical investigations revealed the presence of quercetin in many plant species including *Anaxagorea luzonensis*, which contained a large quantity of this flavonoid (Gonda et al. 2000). Quercetin (**1**) is wide pharmacologically important in terms of anti-inflammatory (Li et al. 2016), anticancer (Zheng et al. 2012), α-glucosidase inhibitory (Li et al. 2009a) activities, and is a beneficial powerful antioxidant (Boots et al. 2008).

In our preliminary investigation on phytochemicals with HbF induction from Thai medicinal plants, we found that quercetin (**1**) isolated as the major constituent from the active crude EtOAc extract of the heartwood of *A. luzonensis* enhanced HbF expression. However, the activity of this compound was less than those of cisplatin and hemin,

the positive controls. Investigation on the chemical modification of quercetin (**1**) for HbF induction has not been reported. We therefore decided to modify the structure of compound **1** to analogs and the methyl ether analogs were chosen for this study. This report describes the isolation of compound **1** from *A. luzonensis* and structural modification of compound **1** to the methyl ether analogs **2**−**15**, and evaluation of HbF-inducing activity of the parent and the synthesized analogs.

Materials and methods

General

Merck pre-coated silica gel 60 F_{254} plates were used for TLC. Spots on TLC were detected under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating. Column chromatography was carried out using Merck silica gel 60 (<0.063 mm and 0.063−0.200 mm). The solvents used were redistilled commercial grade for chromatography and analytical grade for synthesis. Melting points were determined using an electrothermal melting point apparatus and were uncorrected. IR spectra were obtained using a Perkin-Elmer FT-IR Spectrum spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ASCEND 400 spectrometer, operating at 400 (^{1}H) and 100 (^{13}C) MHz. HR-ESI-TOF-MS spectra were measured with a Bruker micrOTOF QII mass spectrometer.

Plant material

The heartwoods of *A. luzonensis* were purchased from Tai-an-jan herbal store, Bangkok, Thailand, in February 2013. A voucher specimen (Apichart Suksamrarn, No. 079) is deposited at the Faculty of Science, Ramkhamhaeng University.

Extraction and isolation of quercetin

The dried heartwoods of *A. luzonensis* (10.0 kg) were milled and macerated successively with *n*-hexane and EtOAc to yield, after evaporation of the solvents under reduced pressure, the *n*-hexane (110.0 g) and EtOAc (995.0 g) extracts, respectively.

The EtOAc extract (990.0 g) was fractionated by column chromatography, using a gradient solvent system of *n*-hexane, *n*-hexane−EtOAc, EtOAc, EtOAc−MeOH and MeOH with increasing amounts of the more polar solvent. The eluates were examined by TLC, and ten combined fractions (E1−E10) were obtained. Fraction E10 (130.1 g) was subjected to column chromatography, using CH_2Cl_2, CH_2Cl_2−MeOH and MeOH with increasing amounts of the more polar solvent to give two subfractions (E10.1−E10.2). Subfraction E10.2 (5.0 g) upon standing, a yellow solid separated out, which was rechromatographed eluting under isocratic condition of 6% MeOH in CH_2Cl_2 to yield **1** (3.5 g) as pale yellow powder.

Quercetin (**1**): pale yellow powder; m.p. >300 °C (MeOH) (lit. m.p. 315.0 °C); IR ν_{max} 3356, 3090, 2956, 1654, 1431, 1090, 881, 817, 786 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz,): $\delta = 7.71$ (1H, d, $J = 2.0$ Hz, H-2′), 7.62 (1H, dd, $J = 8.5$, 2.0 Hz, H-6′), 6.88 (1H, d, $J = 8.5$ Hz, H-5′), 6.37 (1H, d, $J = 1.8$ Hz, H-8), 6.18 (1H, d, $J = 1.8$ Hz, H-6); ^{13}C NMR (CD_3OD, 100 MHz,): $\delta = 177.8$ (C, C-4), 166.1 (C, C-7), 163.0 (C, C-5), 158.7 (C, C-9), 149.2 (C, C-4′), 148.5 (C, C-2), 146.7 (C, C-3′), 137.7 (C, C-3), 124.6 (C, C-1′), 122.1 (CH, C-6′), 116.7 (CH, C-2′), 116.5 (CH, C-5′), 104.5 (C, C-10), 99.7 (CH, C-6), 94.9 (CH, C-8); HR-TOF-MS (ESI$^+$): *m/z* 325.0307 $[M + Na]^+$ (calcd for $C_{15}H_{10}O_7Na$, 325.0318). The physical and NMR spectral data were in accordance with those reported in the literature (Mouffok et al. 2012).

Synthesis of quercetin methyl ether analogs

Preparation of mono- and di-*O*-methylated analogs of quercetin (1)

To a mixture of quercetin (**1**) (200 mg, 0.66 mmol) and anhydrous K_2CO_3 (114 mg, 0.82 mmol) in acetone (20 mL) was added CH_3I (40 µL, $d = 2.28$ g/mL, 0.66 mmol) dropwise. The mixture was kept stirring at room temperature for 2 h, and the progress of the reaction was monitored by TLC. A second lot (20 µL, 0.33 mmol) of CH_3I was added and stirring was continued for another 1 h. Water (50 mL) was added and the mixture was extracted with EtOAc. The combined organic layer was washed with water, dried over anhydrous Na_2SO_4, and the solvent was evaporated in vacuo. The crude products were chromatographed on Sephadex LH-20 column eluting with MeOH to give three fractions (A1−A3). Fraction A1 was subjected to column chromatography over silica gel eluted under isocratic condition of 100% CH_2Cl_2 to yield the di-*O*-methyl ethers **4** (34 mg, 25%) and **5** (26 mg, 19%). Fraction A3 was repeatedly chromatographed over silica gel eluted under isocratic condition of CH_2Cl_2−MeOH (10:0.05) to furnish the mono-*O*-methyl ethers **2** (39 mg, 30%) and **3** (25 mg, 19%), based on the unrecovered starting material, and the starting material **1** (80 mg).

7-Mono-O-methylquercetin (**2**): yellow amorphous solid; IR ν_{max} 3449, 3211, 2952, 2848, 1661, 1417, 1096, 932, 814, 786 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz,): $\delta = 7.75$ (1H, d, $J = 2.1$ Hz, H-2′), 7.65 (1H, dd, $J = 8.4$, 2.1 Hz, H-6′), 6.86 (1H, d, $J = 8.4$ Hz, H-5′), 6.58 (1H, d, $J =$

2.1 Hz, H-8), 6.30 (1H, d, $J = 2.1$ Hz, H-6), 3.88 (3H, s, 7-OC$\underline{H}_3$); ^{13}C NMR (CD_3OD, 100 MHz,): $\delta = 177.4$ (C, C-4), 166.9 (C, C-7), 162.2 (C, C-5), 158.0 (C, C-9), 148.8 (C, C-4′), 146.2 (C, C-3′), 137.5 (C, C-2), 130.6 (C, C-3), 124.0 (C, C-1′), 121.6 (CH, C-6′), 116.1 (CH, C-5′), 116.0 (CH, C-2′), 105.3 (C, C-10), 98.4 (CH, C-6), 92.6 (CH, C-8), 56.3 (CH_3, 7-O$\underline{C}H_3$); HR-TOF-MS (ESI$^+$): *m/z* 339.0473 $[M + Na]^+$ (calcd for $C_{16}H_{12}O_7Na$, 339.0475). The NMR spectral data were compatible to those reported previously (Saewan et al. 2011).

3-Mono-O-methylquercetin (**3**): yellow amorphous solid; IR ν_{max} 3308, 3096, 2980, 2841, 1587, 1439, 1032, 1002, 823, 787 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz,): $\delta = 7.60$ (1H, d, $J = 1.6$ Hz, H-2′), 7.51 (1H, dd, $J = 8.4$, 1.6 Hz, H-6′), 6.88 (1H, d, $J = 8.4$ Hz, H-5′), 6.37 (1H, d, $J = 2.4$ Hz, H-8), 6.17 (1H, d, $J = 2.4$ Hz, H-6), 3.76 (3H, s, 3-OC$\underline{H}_3$); ^{13}C NMR (CD_3OD, 100 MHz,): $\delta = 179.9$ (C, C-4), 165.9 (C, C-7), 163.0 (C, C-5), 158.3 (C, C-9), 157.9 (C, C-2), 149.9 (C, C-4′), 146.4 (C, C-3′), 139.4 (C, C-3), 122.8 (C, C-1′), 122.2 (CH, C-6′), 116.4 (CH, C-2′), 116.3 (CH, C-5′), 105.7 (C, C-10), 99.7 (CH, C-6), 94.6 (CH, C-8), 60.4 (CH_3, 3-O$\underline{C}H_3$); HR-TOF-MS (ESI$^+$): *m/z* 339.0478 $[M + Na]^+$ (calcd for $C_{16}H_{12}O_7Na$, 339.0475). The NMR spectral data were in accordance with those of the reported values (Lee et al. 2003).

7,4′-Di-O-methylquercetin (**4**): yellow amorphous solid; IR ν_{max} 3403, 3178, 2945, 2847, 1655, 1432, 1110, 1006, 915, 819, 789 cm^{-1}; ^{1}H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.78$ (1H, dd, $J = 8.6$, 2.1 Hz, H-6′), 7.74 (1H, d, $J = 2.1$ Hz, H-2′), 6.94 (1H, d, $J = 8.6$ Hz, H-5′), 6.46 (1H, d, $J = 2.0$ Hz, H-8), 6.33 (1H, d, $J = 2.0$ Hz, H-6), 3.94 (3H, s, 4′-OC$\underline{H}_3$), 3.85 (3H, s, 7-OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta = 174.7$ (C, C-4), 165.0 (C, C-7), 159.9 (C, C-5), 156.1 (C, C-9), 156.1 (C, C-2), 147.9 (C, C-4′), 144.9 (C, C-3′), 135.4 (C, C-3), 123.3 (C, C-1′), 120.2 (CH, C-6′), 113.2 (CH, C-2′), 109.9 (CH, C-5′), 103.4 (C, C-10), 97.2 (CH, C-6), 91.5 (CH, C-8), 55.3 (CH_3, 4′-O$\underline{C}H_3$), 55.2 (CH_3, 7-O$\underline{C}H_3$); HR-TOF-MS (ESI$^+$): *m/z* 353.0645 $[M + Na]^+$ (calcd for $C_{17}H_{14}O_7Na$, 353.0631). The NMR spectral data were in agreement with those reported in the literature (Haraguchi et al. 1992).

3,7-Di-O-methylquercetin (**5**): yellow amorphous solid; IR ν_{max} 3397, 3177, 2947, 2849, 1657, 1496, 1110, 916, 820, 790 cm^{-1}; ^{1}H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.64$ (1H, d, $J = 2.2$ Hz, H-2′), 7.56 (1H, dd, $J = 8.4$, 2.2 Hz, H-6′), 6.90 (1H, d, $J = 8.4$ Hz, H-5′), 6.61 (1H, d, $J = 1.6$ Hz, H-8), 6.33 (1H, d, $J = 1.6$ Hz, H-6), 3.88 (3H, s, 7-OC$\underline{H}_3$), 3.79 (3H, s, 3-OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta = 179.1$ (C, C-4), 165.9 (C, C-7), 161.6 (C, C-5), 157.3 (C, C-2), 157.1 (C, C-9), 148.6 (C, C-4′), 145.1 (C, C-3′), 138.8 (C, C-3), 121.9 (C, C-1′), 121.7 (CH, C-6′), 115.7 (CH, C-2′), 115.6 (CH, C-5′), 106.0 (C, C-10), 98.1 (CH, C-6), 92.5 (CH, C-8), 60.2 (CH_3, 3-O$\underline{C}H_3$), 56.0 (CH_3, 7-O$\underline{C}H_3$); HR-TOF-MS (ESI$^+$): *m/z* 353.0648 $[M + Na]^+$ (calcd for $C_{17}H_{14}O_7Na$, 353.0631). The NMR spectral data were in agreement with those of the reported values (Valesi et al. 1972; Wang et al. 1989).

Preparation of di- and tri-*O*-methylated analogs of quercetin (1)

Compound **1** (200 mg, 0.66 mmol) was methylated in the same manner to the preparation of compounds **2**−**5** except that more amounts of anhydrous K_2CO_3 (228 mg, 1.65 mmol) and CH_3I (80 µL, 1.32 mmol) were used, followed by further addition of another lot (40 µL, 0.66 mmol) of CH_3I. After the work-up, the crude products were separated on Sephadex LH-20 column eluting with 10% CH_2Cl_2 in MeOH to give five fractions (B1−B5). Fraction B1 was purified by column chromatography over silica gel eluted under gradient solvent system of hexane−EtOAc (5:2 to 100% EtOAc) to afford the tri-*O*-methyl ethers **8** (43 mg, 27%) and **9** (50 mg, 32%), based on the unrecovered starting material. Fractions B3 and B4 were rechromatographed twice over silica gel eluted under gradient solvent system of EtOAc−hexane−CH_2Cl_2 (3:2:2 to 100% EtOAc) to yield the di-*O*-methyl ethers **4** (24 mg, 20%), **5** (20 mg, 13%), **6** (17 mg, 11%), **7** (8 mg, 5%), based on the unrecovered starting material, and the starting material **1** (64 mg).

3,3′-Di-O-methylquercetin (**6**): yellow amorphous solid; IR ν_{max} 3414, 3266, 2917, 2850, 1658, 1433, 1156, 930, 873, 834 cm^{-1}; ^{1}H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.65$ (1H, d, $J = 2.0$ Hz, H-2′), 7.61 (1H, dd, $J = 8.4$, 2.0 Hz, H-6′), 6.99 (1H, d, $J = 8.4$ Hz, H-5′), 6.38 (1H, d, $J = 2.0$ Hz, H-8), 6.23 (1H, d, $J = 2.0$ Hz, H-6), 3.93 (3H, s, 3′-OC$\underline{H}_3$), 3.79 (3H, s, 3-OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta = 177.9$ (C, C-4), 162.8 (C, C-7), 160.9 (C, C-5), 156.1 (C, C-9), 155.1 (C, C-2), 147.6 (C, C-4′), 145.7 (C, C-3′), 137.8 (C, C-3), 121.8 (CH, C-6′), 121.5 (C, C-1′), 113.9 (CH, C-5′), 110.2 (CH, C-2′), 104.5 (C, C-10), 98.1 (CH, C-6), 93.3 (CH, C-8), 59.4 (CH_3, 3-O$\underline{C}H_3$), 55.3 (CH_3, 3′-O$\underline{C}H_3$); HR-TOF-MS (ESI$^+$): *m/z* 353.0620 $[M + Na]^+$ (calcd for $C_{17}H_{14}O_7Na$, 353.0631). The NMR spectral data were in accordance with those reported in the literature (Al-Dabbas et al. 2006).

3,4′-Di-O-methylquercetin (**7**): yellow amorphous solid; IR ν_{max} 3443, 3273, 3078, 2941, 2841, 1651, 1436, 1089, 915, 880, 795 cm^{-1}; ^{1}H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.64$ (1H, dd, $J = 8.5$, 2.0 Hz, H-6′), 7.61 (1H, d, $J = 2.0$ Hz, H-2′), 6.91 (1H, d, $J = 8.5$ Hz, H-5′), 6.34 (1H, d, $J = 2.0$ Hz, H-8), 6.21 (1H, d, $J = 2.0$ Hz, H-6), 3.92 (3H, s, 4′-OC$\underline{H}_3$), 3.77 (3H, s, 3-OC$\underline{H}_3$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta = 178.2$

(C, C-4), 163.5 (C, C-7), 160.9 (C, C-5), 156.4 (C, C-9), 155.3 (C, C-2), 148.9 (C, C-4′), 145.1 (C, C-3′), 138.3 (C, C-3), 122.5 (C, C-1′), 120.9 (CH, C-6′), 114.1 (CH, C-2′), 110.2 (CH, C-5′), 104.7 (C, C-10), 98.5 (CH, C-6), 93.6 (CH, C-8), 59.7 (CH_3, 3-O$\underline{CH_3}$), 55.5 (CH_3, 4′-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 353.0626 $[M + Na]^+$ (calcd for $C_{17}H_{14}O_7Na$, 353.0631). The NMR spectral data were in accordance with the reported values (Kwon and Kim 2003).

3,7,4′-Tri-O-methylquercetin (**8**): yellow amorphous solid; IR ν_{max} 3360, 3003, 2935, 2843, 1645, 1498, 1091, 924, 814, 767 cm^{-1}; ^1H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.57$ (1H, d, $J = 2.0$ Hz, H-2′), 7.57 (1H, dd, $J = 8.8$, 2.0 Hz, H-6′), 6.87 (1H, d, $J = 8.8$ Hz, H-5′), 6.36 (1H, d, $J = 2.0$ Hz, H-8), 6.24 (1H, d, $J = 2.0$ Hz, H-6), 3.86 (3H, s, 4′-OC$\underline{H_3}$), 3.77 (3H, s, 7-OC$\underline{H_3}$), 3.71 (3H, s, 3-OC$\underline{H_3}$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta = 178.6$ (C, C-4), 165.3 (C, C-7), 161.2 (C, C-5), 156.6 (C, C-9), 156.1 (C, C-2), 149.6 (C, C-4′), 145.7 (C, C-3′), 138.7 (C, C-3), 122.8 (C, C-1′), 121.0 (CH, C-6′), 114.7 (CH, C-2′), 110.6 (CH, C-5′), 105.6 (C, C-10), 97.7 (CH, C-6), 92.0 (CH, C-8), 59.9 (CH_3, 3-O$\underline{CH_3}$), 55.6 (CH_3, 7-O$\underline{CH_3}$), 55.6 (CH_3, 4′-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 367.0785 $[M + Na]^+$ (calcd for $C_{18}H_{16}O_7Na$, 367.0788). The NMR spectral data were in agreement with those of the reported values (Karimova et al. 2015).

7,3′,4′-Tri-O-methylquercetin (**9**): yellow amorphous solid; IR ν_{max} 3347, 3089, 2974, 2846, 1646, 1438, 1091, 887, 814, 795 cm^{-1}; ^1H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.74$ (1H, dd, $J = 8.6$, 1.9 Hz, H-6′), 7.69 (1H, d, $J = 1.9$ Hz, H-2′), 6.91 (1H, d, $J = 8.6$ Hz, H-5′), 6.39 (1H, d, $J = 2.0$ Hz, H-8), 6.28 (1H, d, $J = 2.0$ Hz, H-6), 3.90 (3H, s, 3′-OC$\underline{H_3}$), 3.89 (3H, s, 4′-OC$\underline{H_3}$), 3.81 (3H, s, 7-OC$\underline{H_3}$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta = 175.2$ (C, C-4), 165.4 (C, C-7), 160.3 (C, C-5), 156.5 (C, C-9), 150.5 (C, C-4′), 148.5 (C, C-3′), 145.7 (C, C-2), 135.8 (C, C-3), 123.2 (C, C-1′), 121.3 (CH, C-6′), 110.6 (CH, C-5′), 110.3 (CH, C-2′), 103.8 (C, C-10), 97.6 (CH, C-6), 91.9 (CH, C-8), 55.8, 55.7, 55.7 ($3 \times CH_3$, 7-O$\underline{CH_3}$, 3′-O$\underline{CH_3}$, 4′-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 367.0798 $[M + Na]^+$ (calcd $C_{18}H_{16}O_7Na$, 367.0788). The NMR spectral data were in accordance with those reported in the literature (Dong et al. 1999; Shi et al. 2008).

Preparation of higher methylated analogs of quercetin (1)

To a mixture of quercetin (**1**) (200 mg, 0.66 mmol) and anhydrous K_2CO_3 (342 mg, 2.47 mmol) in acetone (20 mL) was added CH_3I (120 µL, $d = 2.28$ g/mL, 1.98 mmol) dropwise. The mixture was kept stirring at room temperature for 2 h and the progress of the reaction was monitored by TLC. A second lot (60 µL, 0.99 mmol) of CH_3I was added and stirring was continued for another 1 h. After the work-up, the impure product was crystallized with MeOH to yield the tetra-*O*-methyl ether **14** (71 mg, 30%), based on the unrecovered starting material. The filtrate was concentrated and separated on Sephadex LH-20 column eluting with 30% CH_2Cl_2 in MeOH to give seven fractions (C1−C7). The combined fraction (fractions C2−C5) was purified by column chromatography using hexane−CH_2Cl_2−EtOAc (10:1:1 to 100% EtOAc) as eluent to afford the tri-*O*-methyl ethers **8** (38 mg, 17%), **9** (47 mg, 21%), and **10** (25 mg, 11%), based on the unrecovered starting material. Purification of fraction C6 using Sephadex LH-20 column eluting with 100% MeOH followed by column chromatography twice eluted under isocratic condition of hexane−CH_2Cl_2−MeOH (2:4:0.05) furnished the tri-*O*-methyl ethers **11** (2 mg, 1%), **12** (14 mg, 6%) and **13** (2 mg, 1%), based on the unrecovered starting material and the starting material **1** (5 mg).

3,7,3′-Tri-O-methylquercetin (**10**): yellow amorphous solid; IR ν_{max} 3359, 3087, 2973, 2843, 1645, 1461, 1091, 957, 825, 796 cm^{-1}; ^1H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.65$ (1H, d, $J = 1.7$ Hz, H-2′), 7.62 (1H, dd, $J = 8.4$, 1.7 Hz, H-6′), 6.97 (1H, d, $J = 8.4$ Hz, H-5′), 6.41 (1H, d, $J = 2.0$ Hz, H-8), 6.31 (1H, d, $J = 2.0$ Hz, H-6), 3.93 (3H, s, 3′-OC$\underline{H_3}$), 3.83 (3H, s, 7-OC$\underline{H_3}$), 3.79 (3H, s, 3-OC$\underline{H_3}$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta = 178.0$ (C, C-4), 164.7 (C, C-7), 160.9 (C, C-5), 156.0 (C, C-9), 155.5 (C, C-2), 147.9 (C, C-4′), 145.9 (C, C-3′), 138.1 (C, C-3), 121.5 (C, C-1′), 122.0 (CH, C-6′), 114.1 (CH, C-5′), 110.4 (CH, C-2′), 105.3 (C, C-10), 97.2 (CH, C-6), 91.6 (CH, C-8), 59.5 (CH_3, 3-O$\underline{CH_3}$), 55.4 (CH_3, 3′-O$\underline{CH_3}$), 55.2 (CH_3, 7-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 367.0781 $[M + Na]^+$ (calcd for $C_{18}H_{16}O_7Na$, 367.0788). The NMR spectral data were in agreement with those reported in the literature (Al-Dabbas et al. 2006).

5,7,3′-Tri-O-methylquercetin (**11**): yellow amorphous solid; IR ν_{max} 3426, 3009, 2974, 2841, 1612, 1496, 1125, 1009, 814, 790 cm^{-1}; ^1H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.77$ (1H, d, $J = 1.0$ Hz, H-2′), 7.67 (1H, br d, $J = 8.3$ Hz, H-6′), 6.96 (1H, d, $J = 8.3$ Hz, H-5′), 6.50 (1H, br s, H-8), 6.30 (1H, br s, H-6), 3.93 (3H, s, 3′-OC$\underline{H_3}$), 3.91 (3H, s, 5-OC$\underline{H_3}$), 3.86 (3H, s, 7-OC$\underline{H_3}$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta =$ 171.9 (C, C-4), 164.3 (C, C-7), 160.3 (C, C-5), 158.6 (C, C-9), 147.3 (C, C-4′), 146.6 (C, C-3′), 142.6 (C, C-2), 137.2 (C, C-3), 122.9 (C, C-1′), 120.9 (CH, C-6′), 114.6 (CH, C-5′), 110.3 (CH, C-2′), 106.1 (C, C-10), 95.6 (CH, C-6), 92.3 (CH, C-8), 56.1 (CH_3, 3′-O$\underline{CH_3}$), 55.9 (CH_3, 5-O$\underline{CH_3}$), 55.7 (CH_3, 7-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 367.0786 $[M + Na]^+$ (calcd for $C_{18}H_{16}O_7Na$, 367.0788). This is the first report on the spectroscopic data of this compound.

3,3′,4′-Tri-O-methylquercetin (**12**): yellow amorphous solid; IR ν_{max} 3475, 3090, 2980, 2845, 1659, 1460, 1093, 916, 867, 767 cm^{-1}; ^{1}H NMR ($CDCl_3 + 5$ drops of CD_3OD, 400 MHz,): $\delta = 7.68$ (1H, dd, $J = 8.5$, 1.8 Hz, H-6′), 7.64 (1H, d, $J = 1.8$ Hz, H-2′), 6.94 (1H, d, $J = 8.5$ Hz, H-5′), 6.36 (1H, brs, H-8), 6.22 (1H, brs, H-6), 3.92 (3H, s, 4′-O$\underline{CH_3}$), 3.91 (3H, s, 3′-O$\underline{CH_3}$), 3.78 (3H, s, 3-O$\underline{CH_3}$); ^{13}C NMR ($CDCl_3 + 5$ drops of CD_3OD, 100 MHz,): $\delta = 178.6$ (C, C-4), 163.6 (C, C-7), 161.4 (C, C-5), 156.8 (C, C-9), 155.7 (C, C-2), 151.2 (C, C-4′), 148.6 (C, C-3′), 138.7 (C, C-3), 122.8 (C, C-1′), 122.1 (CH, C-6′), 111.1 (CH, C-2′), 110.7 (CH, C-5′), 105.1 (C, C-10), 98.8 (CH, C-6), 94.0 (CH, C-8), 60.1 (CH_3, 3-O$\underline{CH_3}$), 55.9 (CH_3, 3′-O$\underline{CH_3}$), 55.9 (CH_3, 4′-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 367.0827 $[M + Na]^+$ (calcd for $C_{18}H_{16}O_7Na$, 367.0788). The NMR spectral data were in accordance with those of the reported values (Daskiewicz et al. 2005).

5,3′,4′-Tri-O-methylquercetin (**13**): yellow amorphous solid; IR ν_{max} 3351, 3009, 2945, 2844, 1611, 1457, 1054, 1008, 816, 790 cm^{-1}; ^{1}H NMR ($CDCl_3 + 0.1$ mL of CD_3OD, 400 MHz,): $\delta = 7.76$ (1H, d, $J = 2.0$ Hz, H-2′), 7.74 (1H, dd, $J = 8.4$, 2.0 Hz, H-6′), 6.95 (1H, d, $J = 8.4$ Hz, H-5′), 6.47 (1H, d, $J = 1.9$ Hz, H-8), 6.29 (1H, d, $J = 1.9$ Hz, H-6), 3.91 (3H, s, 3′-O$\underline{CH_3}$), 3.89 (3H, s, 4′-O$\underline{CH_3}$), 3.89 (3H, s, 5-O$\underline{CH_3}$); ^{13}C NMR ($CDCl_3 + 0.1$ mL of CD_3OD, 100 MHz,): $\delta = 172.3$ (C, C-4), 163.1 (C, C-7), 160.9 (C, C-5), 158.9 (C, C-9), 150.3 (C, C-4′), 148.7 (C, C-3′), 142.6 (C, C-2), 137.3 (C, C-3), 124.0 (C, C-1′), 120.9 (CH, C-6′), 111.0 (CH, C-5′), 110.6 (CH, C-2′), 105.3 (C, C-10), 95.8 (CH, C-6), 95.3 (CH, C-8), 56.0 (CH_3, 4′-O$\underline{CH_3}$), 56.0 (CH_3, 3′-O$\underline{CH_3}$), 55.9 (CH_3, 5-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 367.0798 $[M + Na]^+$ (calcd for $C_{18}H_{16}O_7Na$, 367.0788). The NMR spectral data were in accordance with those of the reported in the literature (Dong et al. 1999).

3,7,3′,4′-Tetra-O-methylquercetin (**14**): white powder; m.p. 159.5−160.5 °C (lit m.p. 158-159 °C); IR ν_{max} 3153, 3004, 2946, 2910, 2839, 1653, 1429, 1093, 953, 820, 771 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz,): $\delta = 12.57$ (1H, s, 5-O$\underline{H}$), 7.69 (1H, dd, $J = 8.5$, 1.7 Hz, H-6′), 7.64 (1H, d, $J = 1.7$ Hz, H-2′), 6.94 (1H, d, $J = 8.5$ Hz, H-5′), 6.40 (1H, d, $J = 2.0$ Hz, H-8), 6.30 (1H, d, $J = 2.0$ Hz, H-6), 3.92 (3H, s, 4′-O$\underline{CH_3}$), 3.92 (3H, s, 3′-O$\underline{CH_3}$), 3.83 (3H, s, 7-O$\underline{CH_3}$), 3.80 (3H, s, 3-O$\underline{CH_3}$); ^{13}C NMR ($CDCl_3$, 100 MHz,): $\delta =$ 178.6 (C, C-4), 165.3 (C, C-7), 161.8 (C, C-5), 156.6 (C, C-9), 155.8 (C, C-2), 151.2 (C, C-4′), 148.6 (C, C-3′), 138.8 (C, C-3), 122.7 (C, C-1′), 122.1 (CH, C-6′), 111.0 (CH, C-2′), 110.7 (CH, C-5′), 105.9 (C, C-10), 97.7 (CH, C-6), 92.1 (CH, C-8), 60.1 (CH_3, 3-O$\underline{CH_3}$), 55.9 (CH_3, 3′-O$\underline{CH_3}$), 55.9 (CH_3, 4′-O$\underline{CH_3}$), 55.7 (CH_3, 7-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 381.0953 $[M + Na]^+$ (calcd for $C_{19}H_{18}O_7Na$, 381.0944). The physical and NMR spectral data were in agreement with those of the reported data (Chu et al. 2004).

Permethylation of quercetin (1)

A mixture of quercetin (**1**) (15 mg, 0.05 mmol), anhydrous potassium carbonate (70 mg, 0.5 mmol) and acetone (6 mL) was heated under reflux for 30 min and the reaction mixture was then cooled to room temperature. CH_3I (2 mL, excess) was added and the reaction mixture was then refluxed for 24 h. The reaction mixture was poured into cold water and the mixture was extracted with EtOAc; the solution was concentrated in vacuo and left to stand. The solid that separated out was filtered and washed with MeOH to give compound **15** (21.8 mg, 84%).

3,5,7,3′,4′-Penta-O-methylquercetin (**15**): orange amorphous solid; IR ν_{max} 2997, 2936, 2841, 1712, 1600, 1106, 895, 862, 767 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz,): $\delta = 7.24$ (1H, d, $J = 2.1$ Hz, H-2′), 7.21 (1H, dd, $J = 8.2$, 2.1 Hz, H-6′), 6.50 (1H, d, $J = 8.2$ Hz, H-5′), 6.02 (1H, d, $J = 2.2$ Hz, H-8), 5.86 (1H, d, $J = 2.2$ Hz, H-6), 3.48 (9H, s, 5-O$\underline{CH_3}$, 3′-O$\underline{CH_3}$, 4′-O$\underline{CH_3}$), 3.42 (3H, s, 7-O$\underline{CH_3}$), 3.39 (3H, s, 3-O$\underline{CH_3}$); ^{13}C NMR ($CDCl_3$, 100 MHz,): $\delta = 173.9$ (C, C-4), 163.8 (C, C-7), 160.8 (C, C-5), 158.6 (C, C-9), 152.4 (C, C-2), 150.6 (C, C-4′), 148.5 (C, C-3′), 141.0 (C, C-3), 123.2 (C, C-1′), 121.5 (CH, C-6′), 111.0 (CH, C-2′), 110.6 (CH, C-5′), 109.3 (C, C-10), 95.6 (CH, C-6), 92.3 (CH, C-8), 59.8 (CH_3, 3-O$\underline{CH_3}$), 56.3 (CH_3, 3′-O$\underline{CH_3}$), 55.9 (CH_3, 4′-O$\underline{CH_3}$), 55.8 (CH_3, 5-O$\underline{CH_3}$), 55.7 (CH_3, 7-O$\underline{CH_3}$); HR-TOF-MS (ESI^+): *m/z* 395.1125 $[M + Na]^+$ (calcd for $C_{20}H_{20}O_7Na$, 395.1101). The NMR spectral data were in accordance with those reported in the literature (Sutthanut et al. 2007).

Cell line and treatment

K562::$\Delta^{G}\gamma$-$^{A}\gamma$EGFP, a stable reporter assay, containing EGFP coding sequence in-frame replacement of the $^{G}\gamma$- and $^{A}\gamma$-globin coding sequence under the control of $^{G}\gamma$-globin promoter in the human-globin cluster was used for screening of γ-globin gene inducer (Vadolas et al. 2004). These cells were cultured in RPMI 1640 supplemented with 20% fetal bovine serum and incubated at 37 °C in 5% CO_2 incubator. Cells were treated with the tested compounds at different concentrations (0.1−30 μM). EGFP expression was analyzed by flow cytometer after 5 days of treatment. The untreated cells and 0.5% DMSO were used as the negative control, and 5 μM cisplatin and 5 μM hemin were used as positive control. The mean fluorescent intensity of EGFP expression was measured and used for calculation of the fold change response to treated and untreated cells. Cell viability was analyzed by the propidium iodide (PI) staining method. Percentage of PI stained cells as dead cells were used to calculate the cell viability. The results of the tested compounds were compared with untreated cells by paired-*T* test to analyze statistical significance in the fold change of

EGFP expression and the percentage of cell viability. P value ≤ 0.05 was considered as statistical significance.

Results and discussion

Chemistry

HbF-inducing drugs are promising alternative therapies for β-thalassemia. Our preliminary investigation on phytochemicals with HbF induction revealed that quercetin (**1**), the major constituent of the heartwood of *A. luzonensis*, enhanced HbF expression. Since the activity of this compound was lower than the positive controls, cisplatin and hemin, we therefore decided to modify the structure **1** to its analogs. We previously discovered that number of free hydroxyl group played an important role in HbF induction (Chokchaisiri et al. 2010; Chaneiam et al. 2013). We therefore planned to prepare analogs of compound **1** with a varying number of free hydroxyl groups and the methyl ether analogs are the analogs of choice. The strategy of this work is to synthesize analogs with varying numbers of methyl ether functions at different positions of the molecule of **1**.

In the previous reports, a number of mono-*O*-methylquercetins have been synthesized by selective methylation of compound **1** (Jurd 1958; Li et al. 2009b). 7-*O*-Methylquercetin (**2**) was synthesized from **1** by a four-step transformation (Li et al. 2011). Moreover, the direct treatment of the flavonoid **1** with methylating agents in the presence of bases has been explored to yield the corresponding methylated analogs with varying number of methyl ether functions (Jurd 1962; Bouktaib et al. 2002; Juvale et al. 2013; Karimova et al. 2015). Since we need a variety of methylated analogs of compound **1** for our HbF-inducing evaluation, the strategy was to prepare as many methylated analogs as possible. Our preliminary investigation on methylation with different reagents and conditions revealed that methylation of compound **1** using methyl iodide in acetone in the presence of anhydrous potassium carbonate gave rise to methyl ether analogs with varying numbers and positions. We therefore used this reagent in our work. Reaction of quercetin (**1**) with CH_3I in acetone and in the presence of anhydrous K_2CO_3 at room temperature (*ca.* 32 °C), using 1.0 equivalence of CH_3I and 1.25 equivalences of K_2CO_3, followed by addition of another half equivalence of CH_3I, afforded the mono-*O*-methyl ether analogs **2** and **3** in 30% and 19% yields, and the di-*O*-methyl ether analogs **4** and **5** in 25% and 19% yields, respectively, based on the unrecovered starting material **1**. The structure of the ether **2** was established by spectroscopic data, especially those of ^1H and ^{13}C NMR data and mass spectral data. Thus, compound **2** showed the $[M + Na]^+$ ion at *m/z* 339.0473 in the HR-TOFMS (ESI^+) consistent with the molecular formula $C_{16}H_{12}O_7$. The presence of a three-proton singlet signal at δ_H 3.87 and δ_C 56.3 in the ^1H and ^{13}C NMR spectra, respectively, indicated that mono-*O*-methylation has taken place. The position of the methyl ether function has been proven to be at the 7-position by 2D NMR experiments. The heteronuclear multiple bond correlation, a long range proton-carbon coupling (HMBC) spectra showed correlations between 7-OCH_3 (δ 3.87) and C-7 (δ 166.9). In addition, the rotating-frame Overhauser enhancement spectroscopy, a proton-proton space relationship (ROESY) experiments revealed the correlations between 7-OCH_3 and H-8 (δ 6.57), thus confirming that methylation has taken place at the 7-hydroxyl group. By the same analogy, the structure of the mono-*O*-methyl ether **3** was established as 3-mono-*O*-methylquercetin.

Similar synthesis procedure was performed, but using 2 equivalences of CH_3I and 2.5 equivalences of anhydrous K_2CO_3, followed by additional 1.0 equivalence of CH_3I, to yield the di-*O*-methyl ether analogs **4**, **5**, **6** and **7** in 20%, 13%, 11%, and 5% yields, respectively, and the tri-*O*-methyl ether analogs **8** and **9** in 27% and 32% yields, respectively, based on the unrecovered starting material **1**. The structure of the di-*O*-methyl ether **4** was elucidated based on spectroscopic data including ^1H, ^{13}C and 2D NMR and mass spectra. Compound **4** displayed the $[M + Na]^+$ ion at *m/z* 353.0645 in the HR-TOFMS (ESI^+) compatible with the molecular formula $C_{17}H_{14}O_7$. The ^1H NMR data were similar to those of the parent compound **1**. The significant difference was the presence of two methoxyl (7-OCH_3 and 4′-OCH_3) signals at δ 3.85 and δ 3.94 which correlated in HMBC spectra to C-7 (δ 165.0) and C-4′ (δ 147.9), respectively. In addition, the positions of two methoxyl groups were also confirmed by the ROESY experiments. Thus, 7-OCH_3 proton signal showed cross peaks with H-6 (δ 6.33) and H-8 (δ 6.46) and 4′-OCH_3 proton signal also showed cross peak with H-5′ (δ 6.94). Compound **4** was thus concluded as 7,4′-di-*O*-methylquercetin. By the same analogy, the structures of the di-*O*-methyl ethers **5**–**7** were established as 3,7-di-*O*-methylquercetin, 3,3′-di-*O*-methylquercetin, and 3,4′-di-*O*-methylquercetin, respectively.

In order to obtain the additional tri-*O*-methyl ether analogs and tetra-*O*-methyl ether analogs, the methylation procedure was performed in similar manner to that of the synthesis of the mono-*O*-methyl ether analogs **2** and **3**, but using 3.0 equivalences of CH_3I and 3.75 equivalences of K_2CO_3, followed by additional 1.5 equivalences of CH_3I, to yield the tri-*O*-methyl ether analogs **8**, **9**, **10**, **11**, **12** and **13** in 17%, 21%, 11%, 1%, 6%, and 1% yields, respectively, and the tetra-*O*-methyl ether analog **14** in 30% yield. The molecular formula of compound **8** was determined as $C_{18}H_{16}O_7$ from its HR-TOFMS (ESI^+) at *m/z* 367.0785

$[M + Na]^+$. The ^1H and ^{13}C NMR data of **8** indicated that a methoxyl group should be placed at the 3-, 7-, and 4′-positions, which was also confirmed by a detailed analysis of the HMBC and ROESY experiments by the same analogy to that of compound **4**. Thus, the structures of the synthesized tri-*O*-methyl ethers were identified as 3,7,4′-tri-*O*-methylquercetin (**8**), 7,3′,4′-tri-*O*-methylquercetin (**9**), 3,7,3′-tri-*O*-methylquercetin (**10**), 5,7,3′-tri-*O*-methylquercetin (**11**), 3,3′,4′-tri-*O*-methylquercetin (**12**) and 5,3′,4′-tri-*O*-methylquercetin (**13**). It should be noted that the unprecedented tri-*O*-methyl ether analogs **11** and **13** were obtained as the minor products. It is uncommon to find products arising from methylation at the least active 5-hydroxyl group while the hydroxyl groups at other positions (3- and 4′-positions for compound **11** and 3- and 7-positions for compound **13**) are free.

The penta-*O*-methyl ether analog was the last methyl ether analog to be synthesized. By performing similar procedure to those of the foregoing reactions, but using excess CH_3I and the reaction was conducted under refluxing acetone, it was surprisingly found that only small quantity of the penta-*O*-methyl ether analog **15** was yielded. Most of the product was found to be the tetra-*O*-methyl ether analog **14**. However, the required penta-*O*-methyl ether analog **15** was eventually successfully synthesized in 84% yield by preheating compound **1** with K_2CO_3 in acetone under reflux, followed by addition of excess CH_3I to the cooled reaction mixture followed by heating the reaction mixture under reflux.

Biological activity

The crude extracts of *A. luzonensis* were screened for HbF enhancing activity by using the K562::$\Delta^G\gamma^A\gamma$EGFP reporter cells. This cell line has previously been shown to respond to known HbF inducers (Vadolas et al. 2004). The result revealed that the crude EtOAc extract exhibited enhanced EGFP expression when compared with negative control, cells treated with 0.5% DMSO (data not shown). This result prompted us to screen individual isolated compound from this extract. Interestingly, we found that the natural quercetin (**1**), the major flavonoid isolated from the crude EtOAc extract, enhanced γ-globin promotor activity in a dose-dependent manner (1.4 ± 0.1 fold change) with 79.2% cell viability at 30 µM when compared with cells treated with 0.5% DMSO (see Table 1). However, this result was less active than the positive controls, cisplatin and hemin. We therefore decided to investigate whether quercetin analogs would give rise to higher EGFP expression and a number of methyl ether analogs **2**−**15** were therefore synthesized. All

Table 1 The effects of quercetin (**1**) and its analogs **2**−**15** on EGFP expression from the K562::$\Delta^G\gamma$-$^A\gamma$EGFP reporter assay[a] and cell viability

Compound	Substituent					Effective dose (µM)[b]	EGFP fold change	% Cell viability
	3	5	7	3′	4′			
1	OH	OH	OH	OH	OH	30	1.4 ± 0.1	79.2 ± 5.1
2	OH	OH	OCH_3	OH	OH	30	1.0 ± 0.1	83.7 ± 2.2
3	OCH_3	OH	OH	OH	OH	20	1.8 ± 0.0	77.4 ± 3.5
4	OH	OH	OCH_3	OH	OCH_3	20	0.9 ± 0.1	92.4 ± 1.4
5	OCH_3	OH	OCH_3	OH	OH	10	1.0 ± 0.1	90.0 ± 1.6
6	OCH_3	OH	OH	OCH_3	OH	30	1.2 ± 0.0	93.4 ± 4.0
7	OCH_3	OH	OH	OH	OCH_3	8	2.6 ± 0.2	68.9 ± 4.4
8	OCH_3	OH	OCH_3	OH	OCH_3	10	1.3 ± 0.1	86.7 ± 2.9
9	OH	OH	OCH_3	OCH_3	OCH_3	10	1.5 ± 0.2	90.1 ± 1.7
10	OCH_3	OH	OCH_3	OCH_3	OH	10	0.8 ± 0.1	89.8 ± 2.8
11	OH	OCH_3	OCH_3	OCH_3	OH	30	1.1 ± 0.1	93.5 ± 2.8
12	OCH_3	OH	OH	OCH_3	OCH_3	30	1.2 ± 0.1	88.8 ± 1.2
13	OH	OCH_3	OH	OCH_3	OCH_3	10	1.0 ± 0.2	89.0 ± 3.4
14	OCH_3	OH	OCH_3	OCH_3	OCH_3	10	1.1 ± 0.1	91.8 ± 2.3
15	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	10	1.2 ± 0.1	90.8 ± 4.1
Untreated cell						–	1.3 ± 0.1	88.9 ± 2.8
DMSO (0.5%)						–	1.0 ± 0.0	91.9 ± 1.4
Cisplatin[c]						5	2.5 ± 0.7	64.7 ± 9.1
Hemin[c]						5	2.4 ± 0.9	76.2 ± 15.2

[a]Data represent the mean ± SD of three independent experiments

[b]Concentrations are those giving the highest HbF-inducing potency

[c]Cisplatin and hemin were used as positive controls

analogs were also evaluated for HbF-inducing activity. The results indicated that compound **7** presented the highest HbF induction against cell line with 2.6 ± 0.2 fold change at 8 µM. The EGFP expression of compound **7** was slightly highly active compared with the positive controls, cisplatin (2.5 ± 0.7 fold change) and hemin (2.4 ± 0.9 fold change) at 5 µM (Table 1). Through the investigation of EGFP expression using a series of quercetin and analogs with different chemical structures, relationship between the structure and the HbF-inducing effect could be drawn from this study. On comparing the activity of compound **1** with that of compound **2**, it is suggested that the methylation of **1** at the 7-position resulted in decreased HbF induction. On the other hand, methylation of **1** at the 3-position to the analog **3** enhanced its activity. Further methylation at the 4′-position of the 3-methyl ether analog **3** to the 3,4′-dimethyl ether analog **7** strongly enhanced its HbF-inducing potency, suggesting that methylation at the 4′-position also enhanced the activity. Additional methylation at the 7-position to the 3,7,4′-trimethyl ether analog **8** resulted in a considerable decrease in activity, suggesting that structural requirements for high HbF-inducing ability are the presence of 3- and 4′-methoxyl groups and that the 7-hydroxyl group should be free. Comparison of the activity of the 3,4′-dimethyl ether analog **7** with that of the 3,3′,4′-trimethyl ether analog **12** indicated that methylation at the 3′-position resulted in decrease in activity. Polymethylation of compound **1** decreased its activity.

The cytotoxicity of compounds **1**−**15** was also examined. At the concentration that gave the highest EGFP-inducing potency, cell viability of most compounds was more than 70% (Table 1). Compound **7** shows the most toxic with 68.9 ± 4.4% cell viability. Cytotoxic effect of compounds **1** and **7** was not significant difference from that of hemin. When compared with cisplatin, compounds **1** and **7** showed less toxic against K562::$\Delta^{G}\gamma^{A}\gamma$EGFP cells. There are reports about cytotoxic effect of quercetin (**1**) in several cancers including breast, lung, nasopharyngeal, kidney, colorectal, prostate, pancreatic, and ovarian cancers (Rauf et al. 2018). Quercetin exhibits direct proapoptotic effects on tumor cells and thus can inhibit the progress of numerous human cancers. While cisplatin is a well-known chemotherapeutic drug that used to treat a number of cancers, its chemotherapy is also associated with substantial side effects that include hepatotoxic, nephrotoxic, cardiotoxic, neurotoxic, and/or hematotoxic damage (Dasari and Tchounwou 2014). The cytotoxic effect of quercetin is significantly less than cisplatin treatment in the same cell line with comparable concentration which is consistent with this study (Daker et al. 2012; Zhang et al. 2015). Although the anticancer effect of quercetin has been documented in numerous in vitro and in vivo studies that involved several cell lines and animal models, the high toxic effect of quercetin against cancer cells is accompanied with little or no side effects or harm to normal cells (Rauf et al. 2018). The K562::$\Delta^{G}\gamma^{A}\gamma$EGFP cell is an erythroleukemia cell line. Thus the cytotoxic effect of quercetin on K562::$\Delta^{G}\gamma^{A}\gamma$EGFP might also be higher than that in normal cells. However, there was no previous report on biological effect and cytotoxicity of compound **7**. Hydroxyurea, the only US Food and Drug Administration approved drug for HbF inducer, was originally approved as an antineoplastic drug for use in multiple cancers including melanoma, ovarian cancer, and chronic myeloid leukemia. Hydroxyurea has severe side effects including leukopenia, neutropenia, thrombocytopenia, and skin rash (Ballas et al. 2013). However, the dose used for treatment of β-thalassemia and sickle cell anemia patients is lower than that of cancers. This indicated that although compounds **1** and **7** exhibit slightly cytotoxic effect on K562 cells, they may serve as natural product-based alternative lead compounds with HbF-inducing agents for the treatment of β-thalassemia patients.

The regulation of γ-globin gene expression is upon several cellular mechanisms both genetic and epigenetic regulation. One of the proposed mechanisms is oxidative stress defensive pathway due to the existence of antioxidant responsive element (ARE) in promoter of γ-globin gene (Macari and Lowrey 2011). The ARE sequence is the specific region in promoter of cell stress response genes which interact with the transcription factor, nuclear factor erythroid 2-related factor 2 (NRF2), and induced cellular defense system from oxidative stress (Zhang 2006). Several compounds that activate the NRF2/ARE pathway such as angelicin (Lampronti et al. 2003) and resveratrol (Rodrigue et al. 2001) have been shown to induce γ-globin gene expression (Kode et al. 2008; McMahon et al. 2001). Moreover, *tert*-butylhydroquinone induced γ-globin gene expression in K562 cell via NRF2/ARE pathway (Macari et al. 2011). Quercetin (**1**), which is an antioxidative stress polyphenol compound, induced *NQO1* gene expression through the NRF2/ARE signaling pathway (Tanigawa et al. 2007). Therefore, the flavonoid **1** and its analogs may induce γ-globin gene expression by signaling through the NRF2/ARE pathway.

Conclusion

In summary, structural modification of natural quercetin (**1**) isolated from *A. luzonensis* heartwood to 14 *O*-methyl ether analogs was achieved. All compounds were evaluated for HbF-inducing activity. The present study demonstrated for the first time that 3,4′-di-*O*-methylquercetin (**7**), the structurally modified quercetin, exerted potent HbF induction 2.6-fold change, which is slightly greater than the positive

controls, cisplatin and hemin. This compound strongly exhibited EGFP expression with low cellular toxicity. Quercetin (**1**) and its methylated analog **7** could be regarded as a potential lead HbF-inducing molecule derived from natural products for β-thalassemia drug development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Al-Dabbas MM, Kitahara K, Suganuma T, Hashimoto F, Tadera K (2006) Antioxidant and α-amylase inhibitory compounds from aerial parts of *Varthemia iphionoides* Boiss. Biosci Biotechnol Biochem 70:2178–2184

Ballas SK, Singh P, Adams-Graves P, Wordell CJ (2013) Idiosyncratic side effects of hydroxyurea in patients with sickle cell anemia. J Blood Disord Transf 4:1000162

Boots AW, GRMM Haenen, Bast A (2008) Health effects of quercetin: from antioxidant tonutraceutical. Eur J Pharmacol 585:325–337

Bouktaib M, Lebrun S, Atmani A, Rolando C (2002) Hemisynthesis of all the *O*-monomethylated analogues of quercetin including the major metabolites, through selective protection of phenolic functions. Tetrahedron 58:10001–10009

Cao A, Galanello R (2010) Beta-thalassemia. Genet Med 12:67–76

Chaneiam N, Changtam C, Mungkongdee T, Suthatvoravut U, Winichagoon P, Vadolas J, Suksamrarn A, Fucharoen S, Svasti S (2013) A reduced curcuminoid analog as a novel inducer of fetal hemoglobin. Ann Hematol 92:379–386

Chokchaisiri R, Chaneiam N, Svasti S, Fucharoen S, Vadolas J, Suksamrarn A (2010) Labdane diterpenes from the aerial parts of C*urcuma comosa* enhance fetal hemoglobin production in an erythroid cell line. J Nat Prod 73:724–728

Chu HW, Wu HT, Lee YJ (2004) Regioselective hydroxylation of 2-hydroxychalcones by dimethyldioxirane towards polymethoxylated flavonoids. Tetrahedron 60:2647–2655

D'Andrea G (2015) Quercetin: a flavonol with multifaceted therapeutic applications? Fitoterapia 106:256–271

Daker M, Ahmad M, Khoo AS (2012) Quercetin-induced inhibition and synergistic activity with cisplatin—a chemotherapeutic strategy for nasopharyngeal carcinoma cells. Cancer Cell Int 12:34

Dasari S, Tchounwou PB (2014) Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol 740:364–378

Daskiewicz JB, Depeint F, Viornery L, Bayet C, Comte-Sarrazin G, Comte G, Gee JM, Johnson IT, Ndjoko K, Hostettmann K, Barron D (2005) Effects of flavonoids on cell proliferation and caspase activation in a human colonic cell line HT29: an SAR study. J Med Chem 48:2790–2804

Dong H, Gou YL, Cao SG, Chen SX, Sim KY, Goh SH, Kini RM (1999) Eicosenones and methylated flavonols from A*momum koenigii*. Phytochemistry 50:899–902

El-Beshlawy A, Hamdy M, El Ghamrawy M (2009) Fetal globin induction in β-thalassemia. Hemoglobin 33:S197–S203

Fard AD, Hosseini SA, Shahjahani M, Salari F, Jaseb K (2013) Evaluation of novel fetal hemoglobin inducer drugs in treatment of β-hemoglobinopathy disorders. Int J Hematol Oncol Stem Cell Res 7:47–54

Gonda R, Takeda T, Akiyama T (2000) Studies on the constituents of *Anaxagorea luzonensis* A. Gray. Chem Pharm Bull 48:1219–1222

Haraguchi H, Hashimoto K, Yagi A (1992) Antioxidative substances in leaves of *Polygonum hydropiper*. J Agric Food Chem 40:1349–1351

Jurd L (1958) Plant polyphenols. V. selective alkylation of the 7-hydroxyl group in polyhydroxyflavones. J Am Chem Soc 80:5531–5536

Jurd L (1962) The selective alkylation of polyphenols. II. methylation of 7-, 4′-, and 3′-hydroxyl groups in flavonols. J Org Chem 27:1294–1297

Juvale K, Stefan K, Wiese M (2013) Synthesis and biological evaluation of flavones and benzoflavones as inhibitors of BCRP/ABCG2. Eur J Med Chem 67:115–126

Karimova ER, Baltina LA, Spirikhin LV, Kondratenko RM, Farkhutdinov RR, Petrova IV (2015) Synthesis and antioxidant activity of quercetin ethers. Chem Nat Compd 51:851–855

Kode A, Rajendrasozhan S, Caito S, Yang SR, Megson IL, Rahman I (2008) Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 294:L478–L488

Kwon YS, Kim CM (2003) Antioxidant constituents from the stem of *Sorghum bicolor*. Arch Pharm Res 26:535–539

Lampronti I, Bianchi N, Borgatti M, Fibach E, Prus E, Gambari R (2003) Accumulation of gamma-globin mRNA in human erythroid cells treated with angelicin. Eur J Haematol 71:189–195

Lee EH, Kim HJ, Song YS, Jin C, Lee KT, Cho J, Lee YS (2003) Constituents of the stems and fruits of O*puntia ficus-indica* var. s*aboten*. Arch Pharm Res 26:1018–1023

Li NG, Shi ZH, Tang YP, Yang JP, Duan JA (2009b) An efficient partial synthesis of 4'-*O*-methylquercetin via regioselective protection and alkylation of quercetin. Beilstein J Org Chem 5:1–5

Li NG, Shi ZH, Tang YP, Yang JP, Lu TL, Zhang F, Huang YW, Wang ZJ, Duan JA (2011) Synthetic studies on the construction of 7-O-methylquercetin through regioselective protection and alkylation of quercetin. Chin Chem Lett 22:5–8

Li YQ, Zhou FC, Gao F, Bian JS, Shan F (2009a) Comparative evaluation of quercetin, isoquercetin and rutin as inhibitors of α-glucosidase. J Agric Food Chem 57:11463–11468

Li Y, Yao J, Han C, Yang J, Chaudhry MT, Wang S, Liu H, Yin Y (2016) Quercetin, inflammation and immunity. Nutrients 8:167

Liu S, McConnell SC, Ryan TM (2013) Erythropoiesis in the absence of adult hemoglobin. Mol Cell Biol 33:2241–2251

Macari ER, Lowrey CH (2011) Induction of human fetal hemoglobin via the NRF2 antioxidant response signaling pathway. Blood 117:5987–5997

McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, Wolf CR, Cavin C, Hayes JD (2001) The Cap'n'-Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. Cancer Res 61:3299–3307

Mouffok S, Haba H, Lavaud C, Long C, Benkhaled M (2012) Chemical constituents of *Centaurea omphalotricha* Coss. & Durieu ex Batt. & Trab. Rec Nat Prod 6:292–295

Rauf A, Imran M, Khan IA, Ur-Rehman M, Gilani SA, Mehmood Z, Mubarak MS (2018) Anticancer potential of quercetin: a comprehensive review. Phytother Res 32:2109–2130

Rodrigue CM, Arous N, Bachir D, Smith-Ravin J, Romeo PH, Galacteros F, Garel MC (2001) Resveratrol, a natural dietary phytoalexin, possesses similar properties to hydroxyurea toward erythroid differentiation. Br J Haematol 113:500–507

Rund D, Rachmilewitz E (2005) β-Thalassemia. N Engl J Med 353:1135–1146

Saewan N, Koysomboon S, Chantrapromma K (2011) Anti-tyrosinase and anti-cancer activities of flavonoids from *Blumea balsamifera* DC. J Med Plant Res 5:1018–1025

Shi S, Zhao Y, Zhou H, Zhang Y, Jiang X, Huang K (2008) Identification of antioxidants from *Taraxacum mongolicum* by high-performance liquid chromatography-diode array detection-radical-scavenging detection-electrospray ionization mass spectrometry and nuclear magnetic resonance experiments. J Chromatogr A 1209:145–152

Sutthanut K, Sripanidkulchai B, Yenjai C, Jay M (2007) Simultaneous identification and quantitation of 11 flavonoid constituents in *Kaempferia parviflora* by gas chromatography. J Chromatogr A 1143:227–233

Tanigawa S, Fujii M, Hou DX (2007) Action of Nrf2 and Keap1 in ARE-mediated *NQO1* expression by quercetin. Free Radic Biol Med 42:1690–1703

Vadolas J, Wardan H, Orford M, Williamson R, Ioannou PA (2004) Cellular genomic reporter assays for screening and evaluation of inducers of fetal hemoglobin. Hum Mol Genet 13:223–233

Valesi AG, Rodriguez E, Vander Velde G, Mabry TJ (1972) Methylated flavonols in *Larrea cuneifolia*. Phytochemistry 11:2821–2826

Wang Y, Hamburger M, Gueho J, Hostettmann K (1989) Antimicrobial flavonoids from *Psiadia trinervia* and their methylated and acetylated derivatives. Phytochemistry 28:2323–2327

Zhang DD (2006) Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab Rev 38:769–789

Zhang X, Guo Q, Chen J, Chen Z (2015) Quercetin enhances cisplatin sensitivity of human osteosarcoma cells by modulating microRNA-217-KRAS axis. Mol Cells 38:638–642

Zheng SY, Li Y, Jiang D, Zhao J, Ge JF (2012) Anticancer effect and apoptosis induction by quercetin in the human lung cancer cell line A-549. Mol Med Rep 5:822–826