Synthesis of regioselectively acylated quercetin analogues with improved antiplatelet activity

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Abstract. The aim of the present study was to report on a complete synthetic approach, namely benzylation-hydrolysis-acylation-hydrogenation, to the synthesis of regioselectively acylated quercetin analogues using low-cost rutin as a starting material. Three quercetin analogues, quercetin-3-O-propionate (Q-pr), quercetin-3-O-butyrate (Q-bu) and quercetin-3-O-valerate (Q-va), containing 3-, 4- and 5-carbon aliphatic acyl chains, respectively, were synthesized and characterized with ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR and mass spectrometry. Compared with quercetin, all three analogues exhibited improved lipophilicity. The lipophilicity of the analogue increased with increasing acyl chain length. Q-va exhibited the highest lipophilicity among the three analogues, but a lower water solubility compared with quercetin. By contrast, Q-pr and Q-bu exhibited 8.2- and 4.7-fold higher water solubility compared with quercetin, respectively. The *in vitro* and *in vivo* studies demonstrated that Q-pr and Q-bu were more effective whereas Q-va was less effective in inhibiting platelet aggregation compared with quercetin. These results indicated that the water solubility and the lipophilicity of the analogues must be improved in order to achieve higher antiplatelet activity, and an optimal acyl chain length is crucial for the synthesized quercetin analogues to be more effective.

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Introduction

Quercetin is the most abundant dietary flavonoid (1). It was previously demonstrated that quercetin exerts an antiplatelet effect that may be utilized to prevent and alleviate cardiovascular diseases (2-8). However, quercetin exhibits low hydrophilicity and low water solubility. In particular, the low water solubility is associated with the limited bioavailability of quercetin, and remains as a major obstacle to its therapeutic applications (9,10). The strong intermolecular packing of planar phenyl and hereto rings among quercetin molecules is presumably attributed to the low solubility of quercetin in water. Acylation of the hydroxyl groups of quercetin by an acyl donor containing a short aliphatic chain is a feasible way for destroying this intermolecular packing, hence improving the solvation of quercetin. However, non-selective acylation of hydroxyl groups may lead to loss of the biological activities of quercetin. The two adjacent hydroxyl groups at C3 and C4 in ring B, and the double bond between C2-C3 and the carbonyl group at C4 in ring C, are the most critical elements for quercetin to be biologically active (11,12). Therefore, to improve quercetin water solubility without compromising its biological activities, these functional groups must be kept intact.

Regioselective acylation of the hydroxyl group at C3 offers a reasonable solution (13,14). Chemo-enzymatic synthesis of regioselectively acylated quercetin has been previously investigated (13-17). However, synthesis methods without using enzymes are desirable, as they may reduce the reaction time and eliminate the need for costly enzymes. Only few studies using enzyme-free synthesis methods for the synthesis of quercetin analogues have been reported to date (18). A major challenge is that quercetin's hydroxyl groups may be randomly acylated, yielding a mixture of products. The aim of the present study was to develop a complete synthetic method (benzylation-hydrolysis-acylation-hydrogenation) for the synthesis of quercetin analogues using low-cost rutin as a starting material. With this method, regioselective acylation of C3 becomes possible as the hydroxyl of C3 is naturally protected with glycoside via a glycosidic bond. Three quercetin analogues were synthesized, quercetin-3-O-propionate (Q-pr), quercetin-3-O-butyrate (Q-bu) and quercetin-3-O-valerate (Q-va), with high yields (Fig. 1), and their water solubility,

lipophilicity and antiplatelet activity were examined against quercetin.

Materials and methods

Chemicals. Rutin (≥98%) was obtained from Nanjing TCM Institute of Chinese Materia Medica (Nanjing, China) and kept at 110°C under 1.3 kPa for 12 h to remove bound water. Propionyl chloride, butyryl chloride and valeroyl chloride (analytical grade) were purchased from Aladdin Industrial Corporation (Shanghai, China). Benzyl bromide was obtained from Sinopharm Chemical Reagent (Shanghai, China) and distilled prior to use. *N,N*-dimethylformamide (DMF), triethylamine (TEA), and dichloromethane (DCM) were purchased form Tianjin Kemiou Chemical Reagent (Tianjin, China) and dehydrated prior to use. Adenosine diphosphate (ADP), arachidonic acid (AA) and platelet-activating factor (PAF) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Palladium on carbon (Pd/C) (10%) was obtained from Sinopharm Chemical Reagent.

Animals. Male New Zealand rabbits (license no. 0017075) and male Wistar rats (250-300 g; license no. 0010061) were obtained from the Experimental Animal Center of Shandong Lukang Pharmaceutical (Jinan, China). The experimental procedures were approved by the Animal Experimentation Ethics Committee of Weifang Medical College and conducted in accordance with the guidelines of the National Health and Medical Research Council of China for the care and use of animals.

Synthesis of acylated quercetin analogues. The synthesis followed a complete chemical synthetic procedure, namely benzylation-hydrolysis-acylation-hydrogenation, using rutin as the starting material.

Step 1. Rutin (24.42 g, 40 mmol) and K_2CO_3 (18.35 g, 133 mmol) were sequentially added to 160 ml of DMF under nitrogen and stirred for 1 h at room temperature (19). Subsequently, benzyl bromide (16 ml, 133 mmol) was added dropwise into the reaction mixture. The reaction mixture was stirred at 40°C for 3 h under nitrogen, and then adjusted to pH 6.0 with 10% (v/v) acetic acid in ice bath. Deionized water (300 ml) was then added to this mixture and the suspension was filtered.

Step 2. The filtered residue was dissolved in 600 ml of 95% (v/v) ethanol at 70°C. Hydrochloric acid [90 ml, 36% (w/w)] was added to the solution and the hydrolysis continued at 70°C for 2 h. The suspension was cooled down to room temperature and then filtered to yield hydrolyzate. The hydrolyzate was washed with ice water until its pH became neutral.

Step 3. The hydrolyzate was dissolved in 250 ml of DCM followed by addition of chloride (propionyl chloride, butyryl chloride or valeroyl chloride) (44 mmol) and TEA (44 mmol). The mixture was stirred at room temperature until thin-layer chromatography (TLC) analysis showed the completeness of the reaction. The reaction mixture was then extracted with 1 mol/l hydrochloric acid, and the organic layer was washed with a saturated aqueous solution of $NaHCO_3$ and deionized water. The pooled extracts were dried over Na_2SO_4 and
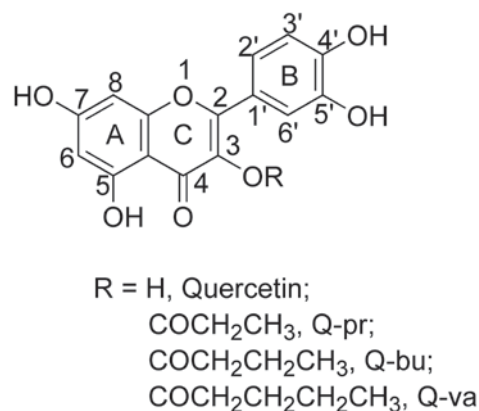

Figure 1. Chemical structures of quercetin and the synthesized quercetin analogues. Q-pr, quercetin-3-*O*-propionate; Q-bu, quercetin-3-*O*-butyrate; Q-va, quercetin-3-*O*-valerate.

then taken to dryness under vacuum to retrieve the acylated compound.

Step 4. The acylated compound was dissolved in 5,000 ml of ethanol/dioxane (1:1, v/v). Pd/C (2 g) was added to the solution. The reaction mixture was stirred at room temperature under hydrogen at atmospheric pressure for 2 h. Pd/C was filtered off, and the filtrate was evaporated in vacuum to retrieve the crude product of acylated quercetin analogues.

Purification of acylated quercetin analogues. Acylated derivatives were purified by using semi-preparative high-performance liquid chromatography (HPLC). An Agilent 1200 infinity preparative chromatography system performed chromatographic separation; this system was equipped with an Agilent Prep LC controller, an ultraviolet (UV) detector (VWD-G1314B), a preparative column (ZORBAX-SB C18, 250x9.4 mm, 5 μm), an injector (3725i) and an auto-fraction collector (G1364C) (all from Agilent Technologies, Inc., Santa Clara, USA). Q-pr and Q-bu were purified using the linear elutions of acetonitrile/water (40:60, v/v and 30:70, v/v, respectively). Q-va was purified using a linear gradient of acetonitrile and water for 0 min (30:70, v/v), 7 min (40:60, v/v), 15 min (50:50, v/v), and 25 min (30:70, v/v). The flow rate was 5 ml/min, and the elution was performed at room temperature at 254 nm with the UV detector.

TLC. The acylation process was monitored using TLC on silica gel 60-GF_{254} (Merck KGaA), with a solvent mixture of ethyl acetate, methanol and acetic acid (at a ratio of 6:4:0.1, v/v/v). The plate was observed and detected under UV light (254 nm).

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Quercetin and three acylated derivatives, Q-pr, Q-bu and Q-va, were characterized with ^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz) in DMSO-d_6 using a Bruker AV500 NMR spectrometer (Bruker Corporation, Billerica, MA, USA) with tetramethylsilane as an internal reference, and with MS without breaking into fragment ions using an Agilent 6410 liquid chromatography-mass spectrometer (Agilent Technologies, Inc.) with electrospray ionization.

Water solubility measurements. The water solubility of quercetin and the three acylated derivatives was determined according to the method described by Bonina *et al* (20). An excess amount of the compound was weighed into a glass tube containing 2 ml of water, and the tube was sealed with a Teflon-lined cap. The mixture was stirred by a magnetic stirrer for 24 h at room temperature and was then filtered using a Millex HV13 filter unit (0.22 μm; Merck KGaA). The drug concentration in the saturated solution at 254 nm was determined using a Shimadzu chromatography system (LC-20AT), equipped with a UV detector (SPD-M10Avp) and a column (Shim-pack VP-ODS C18, 250x4.60 mm, 5 μm) (all from Shimadzu, Kyoto, Japan). The mobile phase was water/methanol (70:30, v/v) at a flow rate of 1 ml/min.

Lipophilicity measurements. Lipophilicity may be estimated using reverse-phase chromatographic retention time due to the good association between the logarithm of the *n*-octanol/water partition coefficient (log P) and the logarithm of the capacity factor eluting with 100% water (log k_w) determined using octadecyl silica columns. The value of log k_w was obtained according to the method described by Braumann (21). Each compound was dissolved in methanol to a final concentration of 10 μg/ml. The sample (n=3) was filtered prior to injection using a filter (Millex HV13, 0.22 μm; Merck KGaA) and the aliquot (20 μl) was injected into the HPLC. The value of logarithm of the capacity factor (log k') was calculated using the following equation:

$$\log k' = \frac{t_r - t_0}{t_0}$$

where t_r is the retention time of the flavonoid peak and t_0 denotes the retention time of the non-retained solvent peak. The HPLC condition was maintained the same as in the water solubility test. Only the volume fraction of methanol in mobile phase was changed for each measurement. A series of values of retention times and log k' were obtained. At a plot of log k' vs. the volume fraction of methanol in mobile phase, φ, extrapolated the data to 100% water to obtain the intersection point with the y axis, that is log k_w value. A formula (21), which exhibited an excellent correlation between log k_w and log P for the compound containing conjugated aromatic, was selected to calculated the value of log P:

$$\log k_w = 0.988 \times \log P + 0.020$$

The lipophilicity of quercetin and the three acylated derivatives was also calculated using the ACD/ChemSketch (Advanced Chemistry Development Inc., Toronto, Canada) and the Property Explorer Applet (www. openmolecules. org).

The association between log k' and φ, the values of log k_w and log P of quercetin and the three acylated derivatives are presented in Table I.

In vitro antiplatelet activity test. Fresh whole blood was collected from healthy male New Zealand rabbits with 3.8% (w/v) sodium citrate at a volume ratio of 9:1. Platelet-rich plasma (PRP) was obtained by centrifugation at 328.7 x g for 8 min at room temperature with no brake. PRP (300 μl) was incubated for 3 min at 37°C in the presence of various final concentrations (0-350 μM) of quercetin or acylated analogue (n=8-10), predissolved in DMSO. The final concentration of DMSO was <1.0% (v/v) to eliminate false positive results. Subsequently, platelet aggregation was stimulated by ADP (7 μM), AA (350 μM), or PAF (7.2 nM). The aggregation was monitored for 5 min using a platelet aggregometer (LBY-NJ4A, Precill, Beijing, China) with constant stirring at 241.5 x g, and the aggregation rates were recorded to determine the percentage of aggregation.

In vivo antiplatelet activity test. Administration of investigational agents and sample preparation for platelet aggregation were performed according to the method described by Mosawy *et al* (22). In brief, the rats (8-10 per group) were treated with 0.03 mmol/kg quercetin, Q-pr, Q-bu, Q-va, or control consisting of 0.5% DMSO with 2.2 mM polyethylene glycol (PEG) in saline. DMSO and PEG were used to improve the solubility of the investigational agents in the blood. The investigational agents were administered via a single intravenous (IV) bolus via the tail vein. Experimental procedures and blood sample collection were performed 30 min after the IV bolus treatment. In each rat, 5 ml of fresh whole blood was collected into tubes containing 600 μl of 3.8% (w/v) sodium citrate via cardiac puncture. PRP was obtained by centrifugation at 377.3 x g for 8 min at room temperature with no brake. PRP was incubated for 3 min at 37°C, and platelet aggregation was then stimulated by 7 μM ADP, 350 μM AA, or 7.2 nM PAF (final concentration). The aggregation was monitored for 5 min by using an aggregometer (LBY-NJ4A, Precill) at a constant stirring of 241.5 x g, and the aggregation rates were recorded to determine the percent of aggregation. The value of the aggregation inhibitory rate (AIR) was calculated as follows:

$$AIR\% = \left(1 - \frac{A}{B}\right) \times 100\%$$

where A is the platelet aggregation rate of the sample and B is the platelet aggregation rate of the no compound treated group (control).

Statistical analysis. All values are expressed as mean ± standard error of the mean. Comparisons between test samples and blank were performed using SPSS 9.0 software for Windows (SPSS Inc., Chicago, IL, USA) with Dunnett's test for post hoc comparisons, and P≤0.01 was considered to indicate statistically significant differences.

Results and Discussion

The synthesis of the three quercetin analogues involves four steps (Fig. 2). The hydroxyl group at C5 is considered non-reactive, as it forms an intramolecular hydrogen bond with the carbonyl at C4 (22). Therefore, this hydroxyl group remained intact throughout the reactions. In step 1, anhydrous K_2CO_3 was added to facilitate the formation of oxygen anions for hydroxyl groups at C3, C4 and C7 of rutin. Subsequently, benzyl bromide was added to benzylate the forming oxygen anions. A protic solvent, DMF, was used to avoid the production of C-alkylated derivatives. The hydroxyl group at C3 was recovered after hydrolysis of the glycoside (step 2), and

Table I. Association between log k' and φ, and values of log k_w, log P and water solubility of quercetin, Q-pr, Q-bu and Q-va.

Compound	log k'-φ (R[a])	log k_w	log P[b] A	B	C	Water solubility (μg/ml)
Quercetin	log k'=1.67–2.16φ (R=-0.98221)	1.67	1.67	2.07	1.49	1.98
Q-pr	log k'=3.04–4.21φ (R=-0.9837)	3.04	3.06	2.77	2.43	16.27
Q-bu	log k'=3.47–4.41φ (R=-0.9943)	3.47	3.48	3.30	2.88	9.36
Q-va	log k'=3.72–4.77φ (R=-0.9995)	3.72	3.75	3.83	3.34	1.07

[a]Linear correlation coefficient. [b]log P was calculated as follows: A, according to the formula log k_w=0.988–log P_{OCT}+ 0.020; B, using the ACD/ChemSketch; C, using the Property Explorer Applet (www.openmolecules.org). Q-pr, quercetin-3-*O*-propionate; Q-bu, quercetin-3-*O*-butyrate; Q-va, quercetin-3-*O*-valerate.

became the only reactive group for subsequent acylation in step 3. In the final step, the benzyl groups at C3, C4 and C7 were removed by hydrogenation. The resulting acylated quercetin derivatives were purified using semi-preparative HPLC. All these derivatives were yellow powders.

The ^{1}H NMR, ^{13}C NMR and MS spectra collectively confirmed the structures of regioselectively acylated quercetin analogues.

Q-pr. ^{1}H NMR chemical shifts: δ (ppm) 12.23 (1H, s, 5-OH), 10.67 (1H, br, s, 7-OH), 9.72 (3H, br s, 3, 3', 4'-OH), 7.33 (1H, d, H-2'), 7.27 (1H, d, H-6'), 6.92 (1H, d, H-5'), 6.48 (1H, d, H-8), 6.25 (1H, d, H-6), 2.68 (2H, t, CH$_2$), 1.15 (3H, t, CH$_3$). ^{13}C NMR chemical shifts: δ (ppm) 175.39 (C-4), 171.78 (C=O), 165.18 (C-7), 161.54 (C-9), 157.08 (C-5), 156.36 (C-2), 149.78 (C-4'), 145.95 (C-3'), 130.06 (C-3), 120.98 (C-1'), 120.08 (C-6'), 116.45 (C-5'), 115.46 (C-2'), 103.89 (C-10), 99.55 (C-6), 94.56 (C-8), 27.08 (CH$_2$), 9.32 (CH$_3$). MS (m/z) for Q-pr: 381.1 [M+Na]$^+$.

Q-bu. ^{1}H NMR chemical shifts: δ (ppm) 12.20 (1H, s, 5-OH), 7.32 (1H, d, H-2'), 7.26 (1H, d, H-6'), 6.91 (1H, d, H-5'), 6.47 (1H, d, H-8), 6.25 (1H, d, H-6), 2.63 (2H, t, CH$_2$), 1.66 (2H, m, CH$_2$), 0.96 (3H, t, CH$_3$). ^{13}C NMR chemical shifts: δ (ppm) 174.95 (C-4), 170.48 (C=O), 164.72 (C-7), 161.08 (C-9), 156.63 (C-5), 156.01 (C-2), 149.31 (C-4'), 145.45 (C-3'), 129.56 (C-3), 120.54 (C-1'), 119.60 (C-6'), 115.95 (C-5'), 115.01 (C-2'), 103.55 (C-10), 99.11 (C-6), 94.12 (C-8), 28.85 (CH$_2$), 17.88 (CH$_2$), 13.33 (CH$_3$). MS (m/z): 395.1 [M+Na]$^+$.

Q-va. ^{1}H NMR chemical shifts: δ (ppm) 12.23 (1H, s, 5-OH), 7.32 (1H, d, H-2'), 7.27 (1H, d, H-6'), 6.92 (1H, d, H-5'), 6.48 (1H, d, H-8), 6.25 (1H, d, H-6), 2.65 (2H, t, CH$_2$), 1.62 (2H, m, CH$_2$), 1.36 (2H, m, CH$_2$), 0.90 (3H, m, CH$_3$). ^{13}C NMR chemical shifts: δ (ppm) 175.40 (C-4), 171.01 (C=O), 165.22 (C-7), 161.55 (C-9), 157.09 (C-5), 156.48 (C-2), 149.77 (C-4'), 145.97 (C-3'), 130.04 (C-3), 122.97 (C-1'), 120.07 (C-6'), 116.38 (C-5'), 115.49 (C-2'), 103.89 (C-10), 99.56 (C-6), 94.56 (C-8), 33.30 (CH$_2$), 26.85 (CH$_2$), 21.93 (CH$_2$), 14.04 (CH$_3$). MS (m/z): 409.1 [M+Na]$^+$.

In the ^{13}C NMR spectrum of quercetin, the chemical shift of C3 appears at 136.18 ppm. However, the chemical shift of C3 in the synthesized analogues moved upfield (130.06 ppm for Q-pr, 129.56 ppm for Q-bu, and130.04 ppm for Q-va). Furthermore, the chemical shifts attributed to the ester carbonyl carbons of Q-pr, Q-bu and Q-va were identified at 171.78, 170.48 and 171.01 ppm, respectively. The ions at m/z

381.1, m/z 395.1 and m/z 409.1 in the MS spectra correspond to sodiated adducts [M+Na]$^+$ for Q-pr, Q-bu and Q-va, respectively, consistent with the C3-substituted structures. This further confirmed the successful substitution of aliphatic acyls at C3.

Q-pr and Q-bu exhibited significantly improved water solubility (Table I). In particular, the water solubility of Q-pr was 8.2-fold higher compared with that of quercetin, while Q-bu exhibited a 4.7-fold higher water solubility. However, Q-va did not exhibit improved water solubility, as its water solubility was only 54% that of quercetin. These results suggested that the water solubility of quercetin analogues is affected by the carbon chain length at C3. Propionyl group has the shortest carbon chain, possessing a total of three carbons. It was shown to be the most effective in increasing water solubility. Butyryl group has one more methylene compared with propionyl group and a total of four carbons. It was still effective in increasing the water solubility of quercetin. However, the five-carbon valeroyl group failed to render the quercetin analogue more water soluble. This was probably because 3- and 4-carbon short aliphatic acyl chains were efficient in disrupting intermolecular packing of quercetin molecules, thus enabling solvent molecules to enter the intermolecular gap to solvate quercetin molecules more easily. A further increase in chain length to 5 carbons made the hydrophobic effect dominant, making quercetin substituted with valeroyl group more hydrophobic. Their *n*-octanol/water partition coefficients (Ps) were tested and three different methods were used to calculate log P (Table I). The lipophilicity of a compound is reflected by log P. Acyl chain length increase resulted in stronger lipophilicity. The results indicate that the log P values of the acylated derivatives were higher than compared with of quercetin, and it increased with aliphatic acyl chain length with log P of Q-va being the highest.

Given that the acylation regioselectively occurred at C3 of quercetin and the bioactive hydroxyl groups remained unchanged, the biological activities of quercetin were expected to be maintained. The *in vitro* assay demonstrated that the three acylated derivatives were able to inhibit platelet aggregation induced by ADP, AA and PAF in a dose-dependent manner (Fig. 3). Q-pr and Q-bu were found to be more potent compared with quercetin.
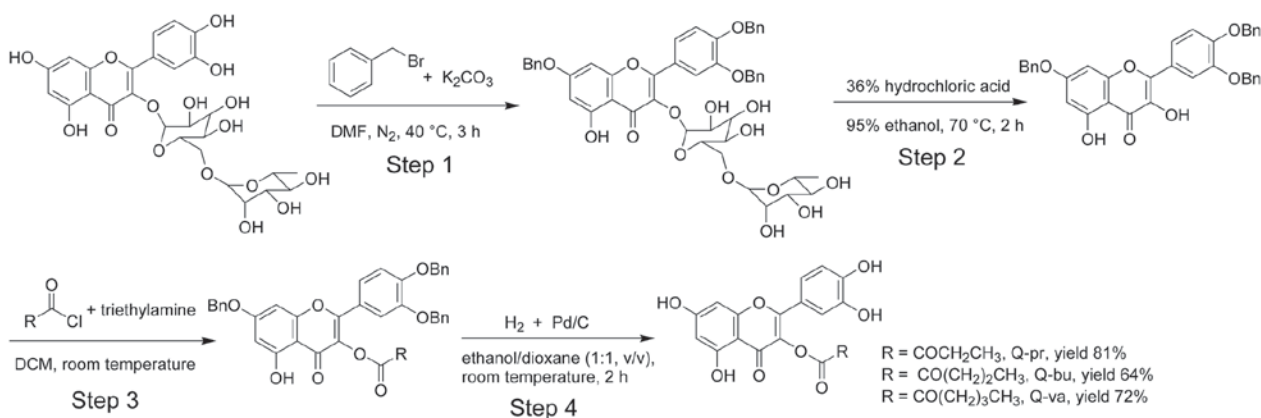
Figure 2. Synthetic approach to the synthesis of regioselectively acylated quercetin analogues. Q-pr, quercetin-3-*O*-propionate; Q-bu, quercetin-3-*O*-butyrate; Q-va, quercetin-3-*O*-valerate; DMF, *N,N*-dimethylformamide; DCM, dichloromethane; Pd/C, palladium on carbon.
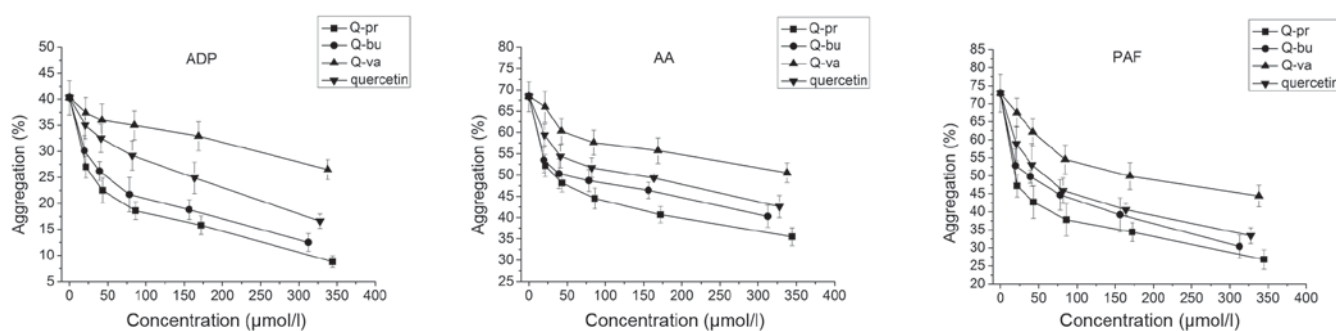

Figure 3. *In vitro* antiplatelet aggregation activities of quercetin, Q-pr, Q-bu, and Q-va. Q-pr, quercetin-3-*O*-propionate; Q-bu, quercetin-3-*O*-butyrate; Q-va, quercetin-3-*O*-valerate; ADP, adenosine diphosphate; AA, arachidonic acid; PAF, platelet-activating factor.
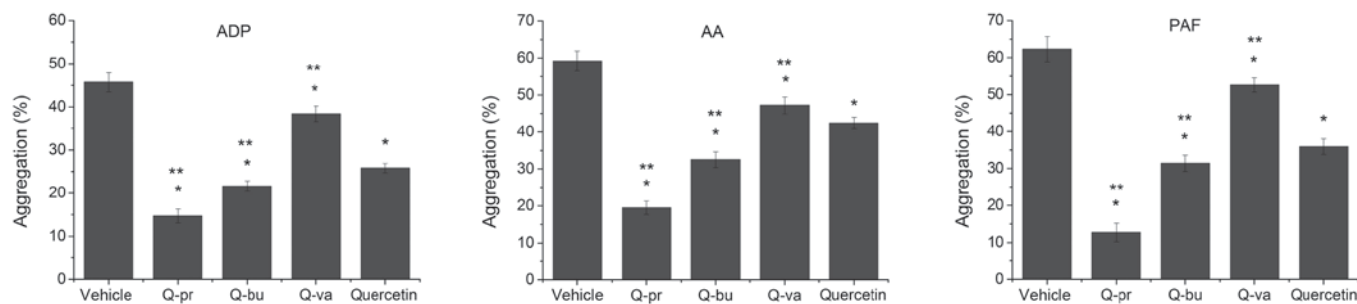

Figure 4. *In vivo* antiplatelet aggregation activities of quercetin, Q-pr, Q-bu, and Q-va. *$P \leq 0.01$ vs. control. **$P \leq 0.01$ vs. quercetin. Q-pr, quercetin-3-*O*-propionate; Q-bu, quercetin-3-*O*-butyrate; Q-va, quercetin-3-*O*-valerate; ADP, adenosine diphosphate; AA, arachidonic acid; PAF, platelet-activating factor.

Q-pr and Q-bu also displayed higher antiplatelet aggregation activities compared with quercetin *in vivo* (Fig. 4). In the ADP group, the AIRs of Q-pr and Q-bu were 67.8 and 52.7%, respectively, which were significantly higher compared with that of quercetin (43.5%). Although Q-va successfully inhibited platelet aggregation to a certain extent with an AIR of 16.0%, it was significantly less potent compared with quercetin. The same order of drug activity of the four compounds was also observed in the AA and PAF groups.

In summary, regioselectively acylated quercetin analogues were successfully synthesized using a complete synthetic approach. The water solubility and lipophilicity of the resulting analogues were affected by the length of the aliphatic chains for acylation of the hydroxyl group located at C3 of quercetin. Aliphatic acyl donors containing three and four carbons were able to improve both the water solubility and the lipophilicity of the acylated analogues. As compared to quercetin, Q-pr and Q-bu exhibited a higher antiplatelet activity due to the higher water solubility and enhanced lipophilicity, whereas Q-va was less effective than quercetin due to its reduced water solubility, although its lipophilicity was the highest among the three analogues. Thus, an optimal acyl chain length is crucial for the quercetin analogues synthesized following this complete synthetic approach to be more effective.

Acknowledgements

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