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Synthesis of organic nitrates of luteolin as a novel class of potent aldose reductase inhibitors

Qi-Qin Wang [a], Ning Cheng [a], Xiao-Wei Zheng [a], Sheng-Ming Peng [a,b,*], Xiao-Qing Zou [a,*]

[a] Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education, College of Chemistry, Xiangtan University, Xiangtan 411105, China
[b] Postdoctoral Programme of Chemical Engineering & Technology, Xiangtan University, Xiangtan 411105, China

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ABSTRACT

Aldose reductase (AR) plays an important role in the design of drugs that prevent and treat diabetic complications. Aldose reductase inhibitors (ARIs) have received significant attentions as potent therapeutic drugs. Based on combination principles, three series of luteolin derivatives were synthesised and evaluated for their AR inhibitory activity and nitric oxide (NO)-releasing capacity in vitro. Eighteen compounds were found to be potent ARIs with IC_{50} values ranging from (0.099 ± 0.008) μM to (2.833 ± 0.102) μM. O^7-Nitrooxyethyl-$O^{3'}$,$O^{4'}$-ethylidene luteolin (**La1**) showed the most potent AR inhibitory activity [IC_{50} = (0.099 ± 0.008) μM]. All organic nitrate derivatives released low concentrations of NO in the presence of L-cysteine. Structure–activity relationship studies suggested that introduction of an NO donor, protection of the catechol structure, and the ether chain of a 2-carbon spacer as a coupling chain on the luteolin scaffold all help increase the AR inhibitory activity of the resulting compound. This class of NO-donor luteolin derivatives as efficient ARIs offer a new concept for the development and design of new drug for preventive and therapeutic drugs for diabetic complications.

1. Introduction

Diabetes mellitus (DM) is a chronic, incurable metabolic disorder defined by the dysregulation of glucose homeostasis manifesting as hyperglycaemia, abnormalities in lipid and protein metabolism, and the development of both acute and long-term complications.[1] According to International Diabetes Federation studies, approximately 366 million people worldwide were diagnosed with diabetes in 2011, and this number is expected to rise to 522 million by 2030.[2] DM is a leading cause of morbidity and mortality in the world, particularly from complications such as macrovascular complications, neuropathy, nephropathy, retinopathy, and cataractogenesis.[3,4] Increasing evidences suggest that aldose reductase may provide a common biochemical link in the pathogenesis of numerous diabetic complications and that the hyperactivity of the polyol metabolic pathway catalysed by AR in individuals with high blood glucose levels contributes to the progression of diabetic complications.[5]

AR is an aldo–keto reductase that catalyses the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of glucose to sorbitol in the first step of the polyol pathway. Sorbitol is subsequently oxidised to fructose by sorbitol dehydrogenase with concomitant reduction of NAD^+ (Fig. 1).[6] Based on these findings, AR has become an attractive molecular target for novel drug design.

ARIs have received attentions as potential therapeutic drugs for the prevention and treatment of diabetic complications.[6,7] Over the last three decades, many compounds with different structures have been reported as ARIs, including alrestatin, tolrestat, epalrestat, zopolrestat, zenarestat, ponalrestat, lidorestat, naphtho[1,2-*d*]isothiazole derivatives, sorbinil, fidarestat, and ranirestat.[8] However, except for epalrestat, none of these compounds are currently marketed. Many of the clinically evaluated ARIs have proven to be inadequate as drug candidates because of their toxic side effects or poor efficacy.[8] Therefore, scientists are exerting much effort into the development of novel ARIs with fewer side effects and excellent efficacy. Interest in flavonoids has steadily increased because of their effectiveness, mild side effects, and relatively low costs.[9–11] A thorough survey of the related literature revealed that flavonoids can modulate the activity of enzymes (such as AR), affect the behaviour of many cell systems, and produce beneficial effects in the body.[12]

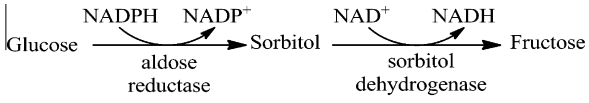

Figure 1. Polyol pathway of glucose metabolism.

* Corresponding authors. Tel.: +86 073158293574.
E-mail address: pengshengming@21cn.com (S.-M. Peng).

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Luteolin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one), a polyphenolic compound available in food products of plant origin, belongs to the flavone subclass of flavonoids and usually appears in its glycosylated form in celery, green pepper, perilla leaf, and camomile tea.[13] Preclinical studies have shown that this flavone possesses a variety of pharmacological activities, including anti-diabetic, by reducing glucose levels, antioxidant, anti-inflammatory, and anticancer activities.[14] Previous reports have established that luteolin shows significant inhibitory activity (IC_{50} = 0.6 μM) against AR.[15–17] Therefore, luteolin, as the scaffold of ARIs, has considerable potential for the treatment of diabetic complications.

NO as a gaseous signalling molecule participates in a plethora of physiological processes, such as regulation of blood pressure, platelet aggregation, neurotransmission, and immune responses.[18,19] Considering the difficulty of performing meaningful biological studies on NO gas, its progenitors (NO donors) are typically utilised in studies that investigate such diverse effects.[18] Previous observations have shown that NO donors inhibit AR activity and sorbitol accumulation in erythrocytes.[20–22] Several studies have demonstrated that inactivation of AR occurs by modification of a hyper-reactive cysteine residue (Cys298) on the active site of AR by thiol-modifying reagents, NO donors, and nitrosothiols.[22,23] Furthermore, the vascular complications of diabetes are closely associated with a decrease in NO generation. Thus, NO donors could supply adequate amounts of NO to prevent AR activity and diabetic complications.

Our recent studies discovered that a derivative of chrysin **I** (Table 1) exhibited in vitro inhibitory activities against AR (IC_{50} = 0.290 ± 0.009 μM) and advanced glycation end-product formation.[24] This derivative of **I** was even observed to increase the glucose consumption of HepG2 cells.[24] Therefore, to study the effect of variations in the lead compound in comparison with chrysin on AR activity, luteolin derivatives were designed as analogues of compound **I**. We postulated that NO donor hybrids that incorporating the active parts of luteolin may be more potent than any of the initial compounds alone. In this study, we coupled NO donors (organic nitrates) to the 7-position of luteolin through a series of ester or ether chains of different spacers (Fig. 2). The NO-releasing capacities and AR inhibitory activities of the resulting derivatives were evaluated in vitro. We believe that this class of NO-donor luteolin compounds is worthy of further study as potential ARIs for inhibiting the polyol pathway and preventing the development of secondary diabetic complications.

2. Chemistry

All derivatives including **La1–6**, **Lb1–6**, and **Lc1–6** described in this study have been obtained by synthesis starting from luteolin, as shown in Schemes 1–4. The preparation of compounds **La1–6** were outlined in Scheme 1. Treating luteolin with 1,2-dibromoethane at 70 °C for 30 min in anhydrous DMF catalyzed by anhydrous K_2CO_3 yielded compound **1**. Compounds **2a–c** were prepared by treating compound **1** with excessive amounts of the appropriate dibromoalkane at reflux (rt) for 2–24 h in anhydrous acetone.[13] These compounds were then reacted with $AgNO_3$ producing products **La1**, **La3**, and **La5**, respectively.[24] Compounds **La2**, **La4**, and **La6** were synthesised according to the method for **2a–c**.

Compounds **Lb1–6** were synthesised in four or five steps from luteolin as shown in Scheme 2.

Compound **1** was reacted with ethyl bromoacetate to afford compound **3**. Subsequent hydrolysis of this compound and reaction with bromoalkane or dibromoalkane produced compounds **Lb2**, **Lb4**, **Lb6**, and **5a–c**. The intermediates **5a–c** were then reacted with $AgNO_3$ producing products **Lb1**, **Lb3**, and **Lb5**, respectively.

The synthetic route for compounds **Lc1**, **Lc3**, and **Lc5** were summarized in Scheme 3. Luteolin was heated with dichlorodiphenylmethane in diphenyl ether at 175 °C for 30 min yielded compound **6**.[25] Compounds **7a–c** were synthesised according to the method for **2a–c**. Intermediates **7a–c** were then reacted with $AgNO_3$ producing compounds **8a–c**. Subsequent cleavage of the diphenylmethyl group of **8a–c** with a mixture of acetic acid and water (4:1) gave the corresponding nitrate derivatives **Lc1**, **Lc3**, and **Lc5**.[26]

Luteolin was treated with 0.5 equiv bromoalkane and anhydrous K_2CO_3 to producing compounds **Lc2**, **Lc4**, and **Lc6**, respectively (Scheme 4).

3. Results and discussion

3.1. Measurement of nitric oxide

Griess assay is the most popular method for the analysis of NO because of its low costs, simple execution, and straightforward data analysis.[27,28] The capacity of thiol-induced NO generation of organic nitrates of luteolin was evaluated after incubation for 1 h in the presence of L-cysteine. The effectiveness of the synthesised compounds was determined with respect to sodium nitroprusside (SNP) as an NO donor. These results are summarised in Table 1.

The percentages of released NO, which varied from 1.018 ± 0.046% to 4.637 ± 0.040%, were equivalent to those of organic nitrates of chrysin.[24] However, the capacity of NO released from SNP was substantially higher (10.42 ± 1.80%) than organic nitrates of luteolin. These results should be evaluated based on the actual additional amount of NO required by the body. The concentrations of NO required to mediate primarily protective effects are extremely low (picomolar to nanomolar range).[24] In the present study, the release of adequate amounts of NO required to protect the body were balanced with the concentration range demanded for the sufficient activity of luteolin derivatives.

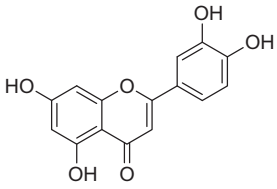
3.2. Aldose reductase inhibitory activity of the target compounds

All newly synthesised derivatives of luteolin were evaluated for their potential inhibitory effect on AR isolated from bovine lenses using quercetin as a reference drug. The assay was based on the spectrophotometric monitoring of NADPH oxidation, which has proven to be a reliable method, with DL-glyceraldehyde as the substrate and NADPH as the cofactor.[24,29] In Table 1, results of the current study were compared with the results previously reported[24] for **I** in a similar assay.

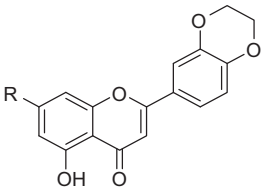
All of the luteolin derivatives exhibited moderate or significant in vitro inhibitory activities on AR with IC_{50} values ranging from (0.099 ± 0.008) μM to (2.833 ± 0.102) μM. Compare with chrysin,[24] the 7-hydroxyl and catechol moiety at the B ring of luteolin could interacts with more AR binding site, therefore, luteolin [(0.754 ± 0.062) μM] exhibited the strong activity. Among the tested compounds, **La1** was the most active ARI, with an IC_{50} value of (0.099 ± 0.008) μM. **La1** was 7.5-fold more potent than luteolin and 28.5-fold more active than quercetin [(2.850 ± 0.040) μM]. These results indicate that replacement of the lead compound with luteolin, as in compounds **La3**, **La1**, and **Lc1**, could improve AR inhibitory activities. **La3** [(0.127 ± 0.011) μM] was 2.3-fold more effective than compound **I** [(0.290 ± 0.009) μM] under the same conditions (Table 1).

Figure 3 shows the AR inhibitory potency of the newly synthesised derivatives and a possible mechanism that explains the structure–activity relationships (SARs) described in the follow section. Figure 3A shows that the AR inhibitory activity of compounds **I**,

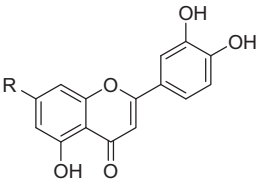
Table 1
In vitro bovine lens AR inhibitory activities and NO-releasing properties of the target compounds



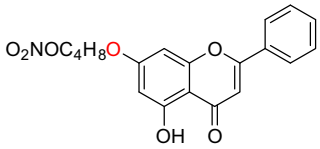
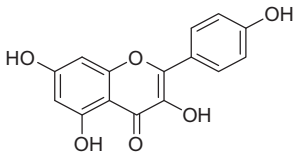
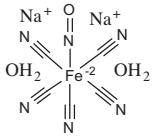
Luteolin



La, Lb



Lc

Compound	Structure	AR inhibition[a]	%No released[b]
0.1% DMSO	R	IC_{50} (μM)	
La1	$NO_3(CH_2)_2O$	0.099 ± 0.008	2.111 ± 0.020
La2	CH_3CH_2O	1.188 ± 0.097	
La3	$NO_3(CH_2)_4O$	0.127 ± 0.011	2.813 ± 0.050
La4	$CH_3(CH_2)_3O$	1.251 ± 0.009	
La5	$NO_3(CH_2)_6O$	0.445 ± 0.036	1.328 ± 0.090
La6	$CH_3(CH_2)_5O$	1.711 ± 0.125	
Lb1	$NO_3(CH_2)_2COOCH_2O$	0.445 ± 0.026	1.018 ± 0.046
Lb2	$CH_3CH_2COOCH_2O$	0.506 ± 0.037	
Lb3	$NO_3(CH_2)_4COOCH_2O$	0.491 ± 0.028	1.351 ± 0.035
Lb4	$CH_3(CH_2)_3COOCH_2O$	0.523 ± 0.045	
Lb5	$NO_3(CH_2)_6COOCH_2O$	1.302 ± 0.086	1.140 ± 0.035
Lb6	$CH_3(CH_2)_5COOCH_2O$	2.833 ± 0.102	
Lc1	$NO_3(CH_2)_2O$	0.168 ± 0.007	1.070 ± 0.035
Lc2	CH_3CH_2O	0.604 ± 0.046	
Lc3	$NO_3(CH_2)_4O$	0.472 ± 0.025	2.345 ± 0.020
Lc4	$CH_3(CH_2)_3O$	0.769 ± 0.067	
Lc5	$NO_3(CH_2)_6O$	0.491 ± 0.032	4.637 ± 0.040
Lc6	$CH_3(CH_2)_5O$	1.810 ± 0.130	
I[24]		0.290 ± 0.009	1.430 ± 0.040
Luteolin		0.754 ± 0.062	
Quercetin[24,c]		2.850 ± 0.040	
SNP[d]			10.420 ± 1.800

Each value represents the mean ± S.D. ($n = 3$).
[a] The concentration required for a 50% inhibition of the decrease in the optical density of NADPH at 340 nm relative to 0.1% DMSO. IC_{50} values were calculated from the dose inhibition curve.
[b] Percent of nitric oxide released based on a theoretical maximum release of 1 mol of NO/mol of the target compounds.
[c] Quercetin was used as positive control for AR inhibition test.
[d] SNP (sodium nitroprusside) was used as positive control for NO releasing test.

La3, **Lb3**, and **Lc3** could be related to their capacity to release NO as well as their log*P* (lipophilicity).[30] The log*P* of the compounds was calculated using ChemBioDraw2010 software. Results showed that the higher the values of both log*P* and NO-releasing capacity, the higher the AR inhibitory activity of the compound is. Meanwhile, remain organic nitrate derivatives also showed the trend. It was found that the order of AR inhibition activity for compounds **La1**, **Lb1**, and **Lc1** was shown as **La1** > **Lc1** > **Lb1** (Table 1) evidenced by comparing the lipophilicity (**La1** > **Lc1** > **Lb1**) and the percentage (**La1** > **Lc1** > **Lb1**) of NO release of compounds. Effects of **La5**, **Lb5**, and **Lc5** on AR inhibition activity were in the rank order of **La5** > **Lc5** > **Lb5** (Table 1). Due to the lipophilicity and the percentage of NO release of **Lb5** were lower than **La5** and **Lc5**. Furthermore, **La5** was more potent than **Lc5**, resulting from comprehensive function of the lipophilicity and the percentage of NO release.

Figure 3B shows that the AR inhibitory activity of the derivatives may be attributed to the lipophilic B ring of the derivative becoming deeply trapped in the lipophilic pocket of the AR active site. Such entrapment releases NO, which could react with β-mercaptoethanol to form RSNO (an analogue of S-nitrosoglutathione) that combines with AR-SH (possibly Cys-298) to form a mixed disulphide.[23]

3.3. Structure–activity relationship studies

Although the alkylate derivatives of luteolin (**La2**, **La4**, **La6**, **Lb2**, **Lb4**, **Lb6**, **Lc2**, **Lc4**, and **Lc6**) showed reasonable IC_{50} values, they

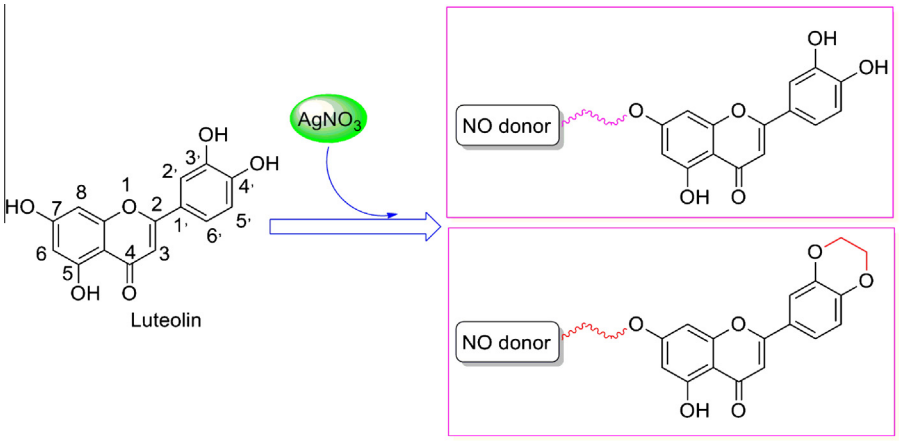
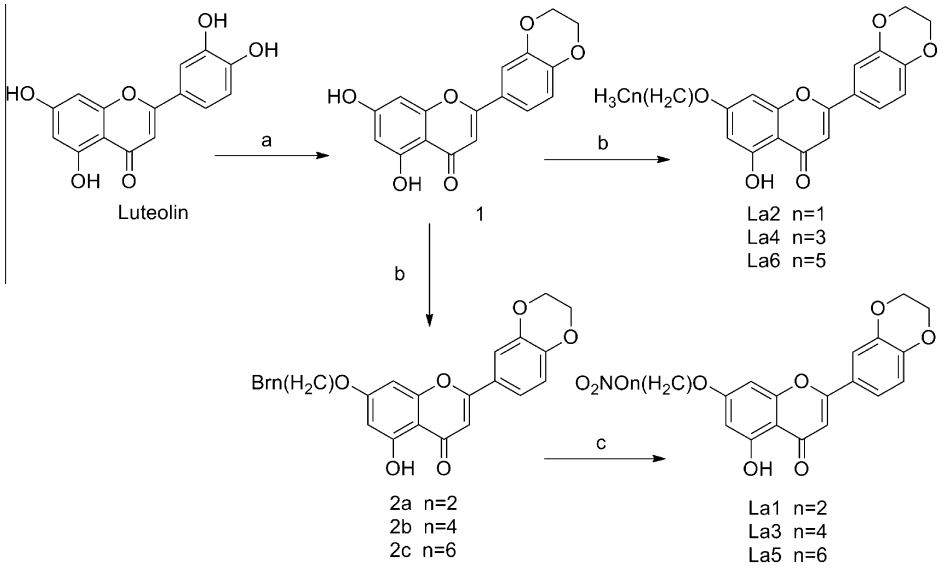


Figure 2. Design concept of novel ARIs based on a luteolin scaffold.



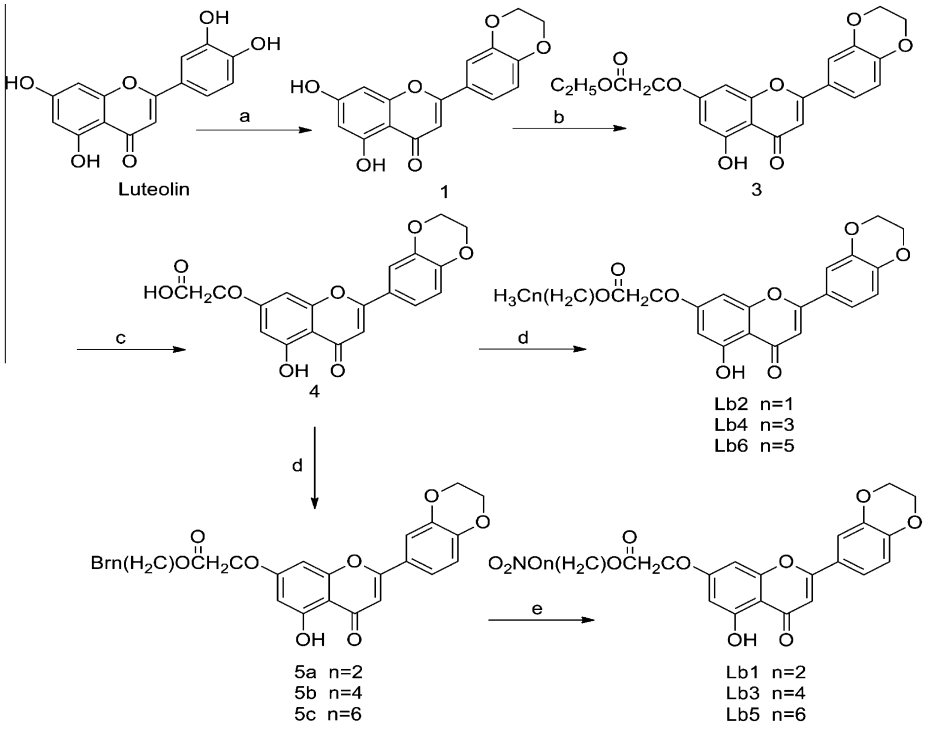
Scheme 1. Preparation of derivatives **La1–6**. Reagents and conditions: (a) DMF, anhydrous K_2CO_3, $BrCH_2CH_2Br$, 70 °C; (b) acetone, anhydrous K_2CO_3, bromoalkane or dibromoalkane, reflux; (c) CH_3CN, THF, $AgNO_3$, 75 °C.

were less effective at AR inhibition than organic nitrate derivatives (Table 1). This phenomenon indicates that inactivation of AR is due in part to the organic nitrates of luteolin releasing low concentrations of NO. In a similar example, Srivastava et al. noted that NO donors generate free NO against AR activity.[22,23] Furthermore, the r^2 value of between NO-releasing capacity and IC_{50} of compounds was 0.623 in the partial correlation analysis (Fig. 3). These results indicate that NO could help increase the AR inhibitory activity of the resulting compound.

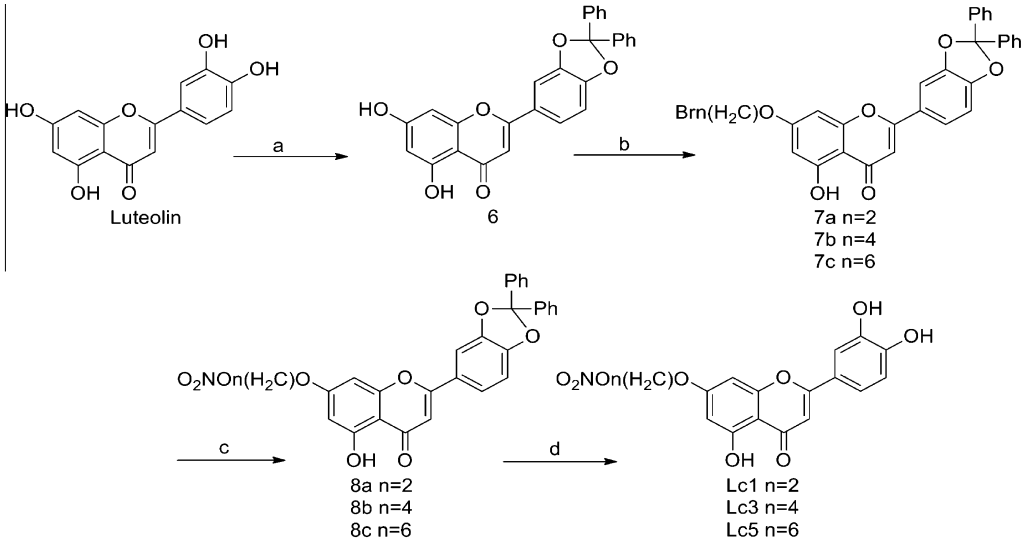
Derivatives in which the B ring of the catechol structure was protected by 1,2-dibromoethane showed appropriately enhanced AR inhibitory activities compared with the corresponding 3′, 4′-unprotected derivatives. This finding was evidenced by comparison of the biological activities of three pairs of derivatives, including **La1** and **Lc1**, **La3** and **Lc3**, and **La5** and **Lc5** (Fig. 4). **La1** was 1.7-fold more potent than **Lc1**. This result may be attributed to the protection provided to the catechol structure, which increases the lipophilicity of the **La** series. Except for **Lc5**, the percentages of NO release by **Lc1** and **Lc3** were lower than those of the corresponding **La** derivatives (Table 1).

Further SAR studies on the importance of the coupling chain of organic nitrates of **La** and **Lb** were conducted. We observed that the organic nitrates of **La** showed enhanced activities in contrast to **Lb**. This finding was evidenced by comparison of the biological activities of three pairs of derivatives, including **La1** and **Lb1**, **La3** and **Lb3**, and **La5** and **Lb5** (Fig. 5). The trends observed may be due to the enhanced ability of the compounds of **La** to release NO compared with the corresponding compounds in **Lb** as well as their increased lipophilicity. Therefore, the AR inhibitory activities of organic nitrate derivatives could be further increased by insertion of an ether chain between the NO donor and luteolin, but the insertion of ester chain shows slight improvement of activities.

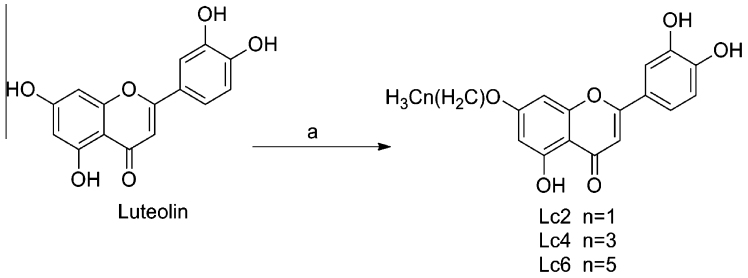
Figures 4 and 5 show that the length of the coupling chain is an important factor in determining AR inhibitory activities. Shorter compounds were more potent than longer ones in each series, as deduced from these figures (**La1** > **La3** > **La5**, **Lb1** > **Lb3** > **Lb5**, and **Lc1** > **Lc3** > **Lc5**). These trends suggest that elongation of the coupling chain reduces the overall inhibition of AR. The ether chain of the 2-carbon spacer as an optimal coupling chain may reasonably be assumed to perfectly fit the nitrooxy moiety at the 7-position of compounds **La1, Lb1**, and **Lc1** near Cys298 (present at the active site of AR) to form RSNO, which would eventually form a mixed disulphide between AR-SH and RSNO.



Scheme 2. Preparation of derivatives **Lb1–6**. Reagents and conditions: (a) DMF, anhydrous K_2CO_3, $BrCH_2CH_2Br$, 70 °C; (b) acetone, anhydrous K_2CO_3, bromo-acetate, rt; (c) (i) KOH, THF, H_2O, 70 °C, (ii) HCl; (d) acetone, anhydrous K_2CO_3, bromoalkane or dibromoalkane, rt; (e) CH_3CN, THF, $AgNO_3$, 75 °C.



Scheme 3. Preparation of derivatives **Lc1**, **Lc3**, and **Lc5**. Reagents and conditions: (a) Ph_2CCl_2, Ph_2O, 175 °C, 30 min; (b) acetone, K_2CO_3, dibromoalkane, rt; (c) CH_3CN, THF, $AgNO_3$, 75 °C; (d) AcOH/H_2O (4:1), rt.



Scheme 4. Preparation of derivatives **Lc2**, **Lc4**, and **Lc6**. Reagents and conditions: (a) DMF, anhydrous K_2CO_3, bromoalkane, 70 °C.

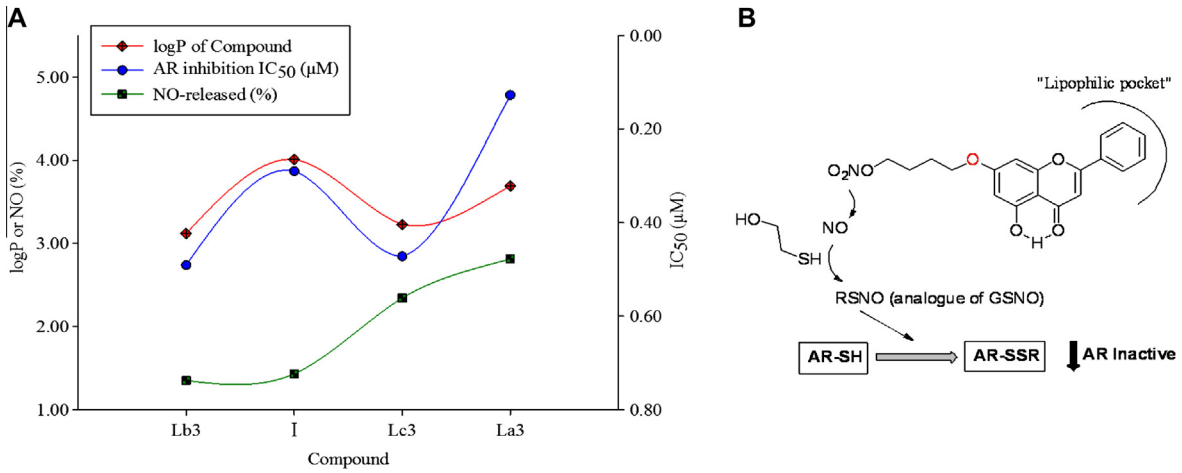


Figure 3. (A) The AR inhibitory activity of compounds **I**, **La3**, **Lb3**, and **Lc3** could be related to their log*P* and NO-releasing capacity. The correlation analysis of these two factors and IC_{50} value should be belong to the partial correlation analysis. The partial correlation coefficient (r) of two variables was calculated using spss16.0 software. The r^2 value of between NO-releasing capacity and IC_{50} of compounds was 0.623, however, the r^2 value of between log*P* and IC_{50} of compounds was 0.767. (B) A possible mechanism of regulation of AR activity by NO-donating derivatives of flavonoids in vitro.

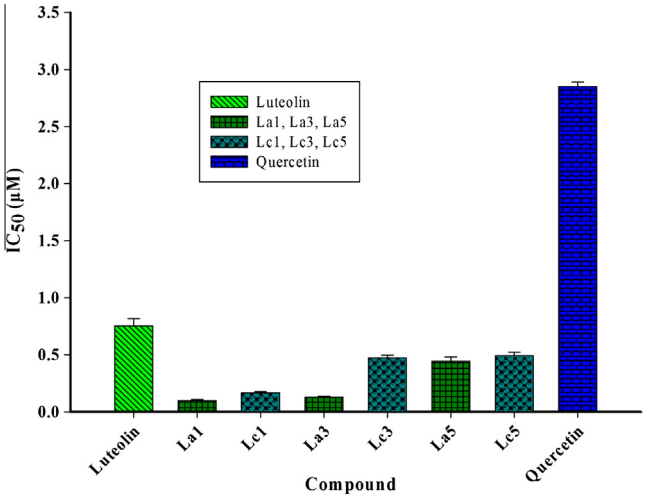


Figure 4. Effect of organic nitrates of **La** and **Lc** on AR activity in vitro.

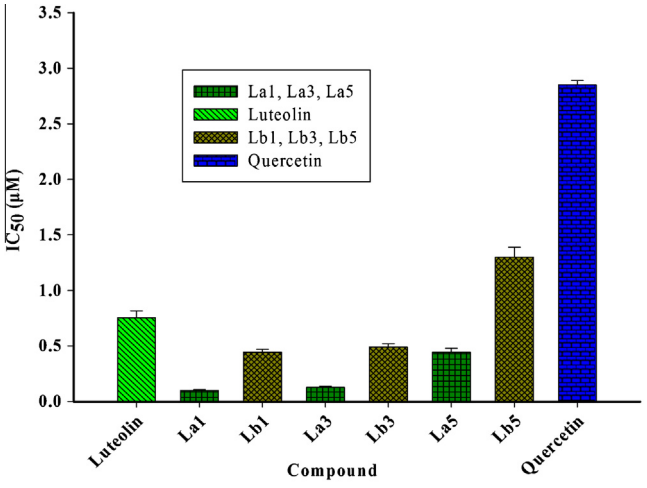


Figure 5. Effect of organic nitrates of **La** and **Lb** on AR activity in vitro.

In conclusion, we described the design, synthesis, evaluation, and SARs of luteolin derivatives bearing nitrooxy alkyl or alkyl groups at the 7-position of luteolin. All of the organic nitrates of luteolin supplied low concentrations of NO to compensate NO in vivo, which could prevent the development of diabetic complications. Alkyl compounds of luteolin exhibited moderate in vitro AR inhibitory activities with IC_{50} values ranging from (0.506 ± 0.037) μM to (2.833 ± 0.102) μM. Organic nitrates of luteolin showed more prominent AR inhibitory activities ranging from (0.099 ± 0.008) μM to (1.302 ± 0.086) μM than alkylate derivatives. **La1** was the most active derivative synthesised and exhibited a 28.5-fold gain in efficacy with respect to the positive reference compound quercetin. We further demonstrated that inactivation of AR may be related to the ability of the derivatives to release NO as well as their lipophilicity. These results encourage us to continue our investigations on the design of more potent ARIs with appropriate modifications based on **La1**. These hybrid ester NO-donor prodrugs as favourable ARIs may provide a new way for preventing and delaying diabetic complications.

4. Experimental section

4.1. General

^{1}H NMR spectra were obtained using a Bruker Avance 400 instrument with $CDCl_3$, DMSO-d_6. TMS was used as an internal standard. Chemical shifts (δ) are reported in ppm and coupling constants (J) are expressed in Hz. Analysis (C, H, N) of the target compounds was performed using an elementary Vario EL III analyser. Molecular masses were determined by matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS) using a Bruker Aupoflex-III mass spectrometer. Melting points (mp) were determined on a Beijing Biotech X-4 micromelting point apparatus. Enzymatic reactions were monitored by a Bio-Tek Synergy HT Multi-Mode microplate reader. All chemicals were of reagent grade and commercially available. Luteolin (>98%) was purchased from Shan Xi Huike Co., Ltd, Jiangsu, China, and used without further purification. When not otherwise specified, anhydrous magnesium sulphate ($MgSO_4$) was used as drying agent for organic phases.

4.2. Synthesis

4.2.1. Experimental procedure for the synthesis of O3′,O4′-ethylidene luteolin (1)

To a solution of luteolin (2.86 g, 10 mmol) in 50 mL of dry DMF was added 1,2-dibromoethane (1.5 mL, 20 mmol) and anhydrous potassium carbonate (0.7 g, 5 mmol), followed by heating at 70 °C for 30 min. To the reaction mixture was added ice water, dropwise. The mixture was filtered, washed with water, dried under reduced pressure. The residue was purified with a silica gel column and was eluted with EtOAc/CH_2Cl_2 = 1:8 to afford **1**, yellow powder, yield 28.1%, mp: 307–309 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 12.90 (s, 1H, 5-OH), 10.87 (s, 1H, 7-OH), 7.60–7.57 (m, 2H, $PhH_{2',6'}$), 7.04–7.02 (d, 1H, J = 8 Hz, $PhH_{5'}$), 6.87 (s, 1H, PhH_8), 6.51 (d, 1H, J = 1.7 Hz, PhH_3), 6.20–6.19 (d, 1H, J = 4 Hz, PhH_6), 4.33–4.32 (d, 4H, J = 4 Hz, –CH_2CH_2–). MALDI-TOF: m/z 313 ([M+H]$^+$).

4.2.2. General procedures for the synthesis of compounds La1–6

To a stirred solution of **1** (0.312 g, 1 mmol) in dry acetone (20 mL), anhydrous potassium carbonate (0.14 g, 1 mmol) was added. After 30 min, the appropriate bromo alkane or dibromo alkane (10 mmol) were added; the reaction mixture was stirred at reflux until TLC evidenced complete consumption of starting material (2–24 h). The solvent was removed under reduced pressure (water pump) and the residue was washed with water (3 × 20 mL), then dried. The residue was purified by column chromatography, eluting with CH_2Cl_2 to afford the desired derivatives **2a–c** and **La2**, **4**, **6**.

A solution of the appropriate bromo derivatives **2a–c** (0.5 mmol) and $AgNO_3$ (0.85 g, 5 mmol) in dry CH_3CN (30 mL) and THF (10 mL) was stirred at 75 °C. The reaction was allowed to proceed for 2–8 h in the dark. Filtration was performed to remove any silver bromide precipitates. The filtrate was concentrated under reduced pressure and a yellow solid was produced. This solid was washed with water until the runoff was clear. Solid samples were dried and purified by a silica gel column, eluting with CH_2Cl_2 to afford the final products **La1**, **3**, **5**.

4.2.2.1. O7-Bromethyl-O3′,O4′-ethylidene luteolin (2a). 1,2-Dibromoethane (0.87 mL, 10 mmol), yellow powder, yield: 64%, mp: 218–220 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.56 (s, 1H, aromatic H_8), 6.48 (s, 1H, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.37–4.33 (m, 6H, CH_2, –CH_2CH_2–), 3.68–3.65 (t, 2H, J = 6 Hz, –CH_2Br). MALDI-TOF: m/z 419 ([M+H]$^+$).

4.2.2.2. O7-Brombutyl-O3′,O4′-ethylidene luteolin (2b). 1,4-Dibromobutane (1.22 mL, 10 mmol), yellow solid, yield: 52.8%, mp: 156–158 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.98–6.96 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.54 (s, 1H, aromatic H_8), 6.46 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.33–4.32 (d, 4H, J = 4 Hz, –CH_2CH_2–), 4.08–4.06 (t, 2H, J = 4 Hz, –OCH_2–), 3.51–3.48 (t, 2H, J = 6.1 Hz, –CH_2Br), 2.09–1.99 (m, 4H, –CH_2CH_2–). MALDI-TOF: m/z 447 ([M+H]$^+$).

4.2.2.3. O7-Bromhexyl-O3′,O4′-ethylidene luteolin (2c). 1,6-Dibromohexane (1.56 mL, 10 mmol), yellow powder, yield: 68%, mp: 149–151 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 7.41–7.38 (m, 2H, aromatic $H_{2',6'}$), 6.98–6.96 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.54 (s, 1H, aromatic H_8), 6.46 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.33 (d, 4H, J = 2.4 Hz, –CH_2CH_2–), 4.04–4.02 (t, 2H, J = 4 Hz, –OCH_2–), 3.45–3.42 (t, 2H, J = 6.3 Hz, –CH_2Br), 1.91–1.83 (m, 4H, CH_2, CH_2), 1.52(s, 4H, –CH_2CH_2–). MALDI-TOF: m/z 475 ([M+H]$^+$).

4.2.2.4. O7-Nitrooxyethyl-O3′,O4′-ethylidene luteolin (La1). Compound **2a** (210 mg, 0.5 mmol), yellow powder, yield: 67.5%, mp: 151–153 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.88 (s, 1H, 5-OH), 7.62–7.59 (m, 2H, aromatic $H_{2',6'}$), 7.04–7.02 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.93 (s, 1H, aromatic H_8), 6.86 (s, 1H, aromatic H_3), 6.40 (s, 1H, aromatic H_6), 4.91 (s, 2H, –CH_2ONO_2), 4.45 (s, 2H, –CH_2–), 4.34–4.32 (d, 4H, J = 8 Hz,–CH_2CH_2–). MALDI-TOF: m/z 424 ([M+Na]$^+$). Anal. Calcd for $C_{19}H_{15}NO_9$: C, 56.86; H, 3.77; N, 3.49. Found: C, 56.53; H, 3.72; N, 3.30.

4.2.2.5. O7-Ethyl-O3′,O4′-ethylidene luteolin (La2). Bromoethane (0.75 mL, 10 mmol), yellow powder, yield: 69%, mp: 173–175 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.98–6.96 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.54 (s, 1H, aromatic H_8), 6.45 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.33–4.32 (d, 4H, J = 4 Hz, –CH_2CH_2–), 4.13–4.08 (q, 2H, J = 6.7 Hz, –CH_2–), 1.47–1.43 (t, 3H, J = 8 Hz, –CH_3). MALDI-TOF: m/z 341 ([M+H]$^+$).

4.2.2.6. O7-Nitrooxybutyl-O3′,O4′-ethylidene luteolin (La3). **2b** (224 mg, 0.5 mmol), yellow powder, yield: 86%, mp: 150–153 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.86 (s, 1H, 5-OH), 7.62–7.59 (m, 2H, aromatic $H_{2',6'}$), 7.04–7.02 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.91 (s, 1H, aromatic H_8), 6.81 (s, 1H, aromatic H_3), 6.36 (s, 1H, aromatic H_6), 4.60 (s, 2H, –CH_2ONO_2), 4.34–4.32 (d, 4H, J = 8 Hz, –CH_2CH_2–), 4.14 (s, 2H, –CH_2O–), 1.83 (s, 4H, –CH_2CH_2–). MALDI-TOF: m/z 452 ([M+Na]$^+$). Anal. Calcd for $C_{21}H_{19}NO_9$: C, 58.74; H, 4.46; N, 3.26. Found: C, 58.50; H, 4.08; N, 3.14.

4.2.2.7. O7-Butyl-O3′,O4′-ethylidene luteolin (La4). Bromobutane (1.07 mL, 10 mmol), yellow powder, yield: 79%, mp: 154–156 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.98–6.96 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.54 (s, 1H, aromatic H_8), 6.45 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.33–4.32 (d, 4H, J = 4 Hz, –CH_2CH_2–), 4.04–4.01 (t, 2H, J = 6.2 Hz, –CH_2O–), 1.81–1.78 (t, 2H, J = 7 Hz, –CH_2–), 1.53–1.47 (q, 2H, J = 7.2 Hz, –CH_2–), 1.01–0.97 (t, 3H, J = 7.2 Hz, –CH_3). MALDI-TOF: m/z 369 ([M+H]$^+$).

4.2.2.8. O7-Nitrooxyhexyl-O3′,O4′-ethylidene luteolin (La5). **2c** (238 mg, 0.5 mmol), yellow powder, yield: 75%, mp: 156–158 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 12.85 (s, 1H, 5-OH), 7.62–7.59 (m, 2H, aromatic $H_{2',6'}$), 7.04–7.02 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.90 (s, 1H, aromatic H_8), 6.80 (s, 1H, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.54–4.51 (t, 2H, J = 6.2 Hz, –CH_2ONO_2), 4.34–4.32 (d, 4H, J = 8 Hz, –CH_2CH_2–), 4.10–4.08 (t, 2H, J = 4 Hz, –CH_2O–), 1.75–1.68 (m, 4H, CH_2, CH_2), 1.43 (s, 4H, –CH_2CH_2–). MALDI-TOF: m/z 480 ([M+Na]$^+$). Anal. Calcd for $C_{23}H_{23}NO_9$: C, 60.39; H, 5.07; N, 3.06. Found: C, 59.98; H, 4.98; N, 2.95, aromatic.

4.2.2.9. O7-Hexyl-O3′,O4′-ethylidene luteolin (La6). Bromohexane (1.42 mL, 10 mmol), yellow powder, yield: 68.7%, mp: 125–127 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.40–7.36 (m, 2H, aromatic $H_{2',6'}$), 6.97–6.95 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.52 (s, 1H, aromatic H_8), 6.44 (s, 1H, aromatic H_3), 6.33 (s, 1H, aromatic H_6), 4.32–4.31 (d, 4H, J = 4 Hz, –CH_2CH_2–), 4.03–4.00 (t, 2H, J = 6.5 Hz, –CH_2O–), 1.82–1.78 (t, 2H, J = 8 Hz, –CH_2–), 1.46–1.34 (m, 6H, –$CH_2CH_2CH_2$–), 0.91 (s, 3H, –CH_3). MALDI-TOF: m/z 397 ([M+H]$^+$).

4.2.3. General procedures for the synthesis of compounds Lb1–6

4.2.3.1. O7-(Ethoxycarbonyl)methyl-O3′,O4′-ethylidene luteolin (3). Anhydrous potassium carbonate (10 mmol, 1.38 g) was added to a stirred solution of **1** (10 mmol, 3.12 g) in dry acetone

(150 mL), followed by refluxing until the solution became clear. Ethyl bromoacetate (30 mmol, 3.5 mL) was then dribbled into the mixture, and acetone (10 mL) was added. The solution was refluxed for 4 h and vacuum filtered. The filter liquor was concentrated to obtain a yellow solid, which was subsequently washed with petroleum, water. The solid was then dried and dissolved in dichloromethane. Any undissolved solid were discarded. The solution was concentrated and dried to produce a yellow powder **3**, yield: 79.6%, mp: 133–135 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.81 (s, 1H, 5-OH), 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.55 (s, 1H, aromatic H_8), 6.48–6.47 (d, 1H, J = 4 Hz, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.70 (s, 2H, –CH_2O–), 4.33–4.32 (d, 4H, J = 4 Hz, –CH_2CH_2–), 3.83 (s, 2H, –CH_2O-CO–), 1.34–1.30 (t, 3H, J = 7.2 Hz, –CH_3).

4.2.3.2. O7-Carboxymethyl-O3′,O4′-ethylidene luteolin (4). The solution of **3** (5 mmol, 1.99 g) in THF (25 mL), H_2O (100 mL) and ethanol (200 mL) was stirred at 70 °C for 15 min, and then a solution of potassium hydroxide (30 mmol, 1.68 g) in H_2O was added dropwise. The reaction mixture was stirred at 70 °C until TLC evidenced complete consumption of starting material (8 h). The resulting homogeneous solution was concentrated and dissolved in H_2O. Any undissolved solid were discarded. Then, the pH was adjusted to the desired acidity with hydrochloric acid. The solution underwent vacuum filtration, as well as water washing and drying to afford a yellow solid **4**, yield: 52.7%, mp: 320–322 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 12.87 (s, 1H, 5-OH), 7.64–7.60 (m, 2H, aromatic $H_{2',6'}$), 7.04–7.02 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.94 (s, 1H, aromatic H_8), 6.80 (s, 1H, aromatic H_3), 6.36 (s, 1H, aromatic H_6), 4.77 (s, 2H, –CH_2O–), 4.34–4.32 (d, 4H, J = 8 Hz, –CH_2CH_2–).

4.2.3.3. O7-[(Bromoethoxyl)carbonyl]methyl-O3′,O4′-ethylidene luteolin (5a–c, Lb2, Lb4 and Lb6). Triethylamine (18 mmol, 2.55 mL) was added to a solution of **4** (3 mmol, 1.11 g) in DMSO (5 mL) and acetone (100 mL). The reaction mixture was then refluxed for 30 min. 1,2-Dibromoethane (15 mmol, 1.31 mL) was dribbled into the solution, followed by refluxing for 24 h and filtration to remove any precipitates that may have formed. The filter liquor was concentrated. The residue was added ice water to produce a yellow solid. The solid was filtered and dried to obtain desired compound **5a**, yield: 73.2%, mp: 193–195 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.81 (s, 1H, 5-OH), 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.98–6.96 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.55 (s, 1H, aromatic H_8), 6.49 (s, 1H, aromatic H_3), 6.36 (s, 1H, aromatic H_6), 4.75 (s, 2H, –CH_2O–), 4.56–4.53 (t, 2H, J = 5.6 Hz, –CH_2–), 4.33–4.32 (d, 4H, J = 4 Hz, –CH_2CH_2–), 3.56–3.54 (t, 2H, J = 8 Hz, –CH_2Br).

Compounds **5b–c**, **Lb2**, **Lb4** and **Lb6** were synthesized according to the method for **5a**.

*Compound **5b***: 1,4-Dibromobutane (15 mmol, 1.83 mL), yield: 78.7%, mp: 167–169 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 12.81 (s, 1H, 5-OH), 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.55 (s, 1H, aromatic H_8), 6.48 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.70 (s, 2H, –CH_2O–), 4.33–4.25 (m, 6H, –CH_2CH_2–, –CH_2OCO–), 3.42–3.39 (t, 2H, J = 6 Hz, –CH_2Br), 1.91–1.84 (m, 4H, –CH_2CH_2–).

*Compound **5c***: 1,6-Dibromohexane (15 mmol, 2.34 mL), yield: 73.6%, mp: 155–157 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.81 (s, 1H, 5-OH), 7.41–7.40 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.56 (s, 1H, aromatic H_8), 6.48 (s, 1H, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.70 (s, 2H, –CH_2O–), 4.33–4.22 (m, 6H, CH_2CH_2, –CH_2OCO–), 3.40–3.36 (t, 2H, J = 6.4 Hz, –CH_2Br), 1.85–1.67 (m, 4H, CH_2, CH_2), 1.45–1.36 (m, 4H, –CH_2CH_2–).

*Compound **Lb2***: Bromoethane (15 mmol, 1.13 mL), yield: 79.6%, mp: 133–135 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 12.81 (s, 1H, 5-OH), 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.55 (s, 1H, aromatic H_8), 6.48–6.47 (d, 1H, J = 4 Hz, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.70 (s, 2H, –CH_2O–), 4.33–4.32 (d, 4H, J = 4 Hz, –CH_2CH_2–), 3.83 (s, 2H, –CH_2OCO–), 1.34–1.30 (t, 3H, J = 7.2 Hz, –CH_3). MALDI-TOF: m/z 399 ([M+H]$^+$).

*Compound **Lb4***: Bromobutane (15 mmol, 1.61 mL), yield: 88.6%, mp: 141–143 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 12.81 (s, 1H, 5-OH), 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.55 (s, 1H, aromatic H_8), 6.48 (s, 1H, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.69 (s, 2H, –CH_2O–), 4.33 (s, 4H, –CH_2CH_2–), 4.25–4.22 (t, 2H, J = 6.1 Hz, –CH_2OCO–), 1.67–1.64 (t, 2H, J = 6.8 Hz, –CH_2–), 1.40–1.35 (q, 2H, J = 7.1 Hz, –CH_2–), 0.94–0.91 (t, 3H, J = 7 Hz, –CH_3). MALDI-TOF: m/z 427 ([M+H]$^+$).

*Compound **Lb6***: Bromohexane (15 mmol, 2.13 mL), yield: 85.2%, mp: 110–112 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 12.80 (s, 1H, 5-OH), 7.41–7.38 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.56 (s, 1H, aromatic H_8), 6.48 (d, 1H, J = 1.6 Hz, aromatic H_3), 6.35 (d, 1H, J = 1.5 Hz, aromatic H_6), 4.69 (s, 2H, –CH_2O–), 4.34–4.33 (d, 4H, J = 4 Hz, –CH_2CH_2–), 4.24–4.21 (t, 2H, J = 6.6 Hz, –CH_2OCO–), 1.68–1.62 (m, 2H, –CH_2–), 1.29–1.25 (m, 6H, –$CH_2CH_2CH_2$–), 0.87–0.85 (d, 3H, J = 8 Hz, –CH_3). MALDI-TOF: m/z 458 ([M+H]$^+$).

4.2.3.4. O7-[(Nitrooxyl)ethoxycarbonyl]methyl-O3′,O4′-ethylidene luteolin (Lb1, Lb3, Lb5). Compounds **Lb1**, **Lb3** and **Lb5** were synthesized according to the method for **La1**.

*Compound **Lb1***: Compound **5a** (239 mg, 0.5 mmol), $AgNO_3$ (0.85 g, 5 mmol), yield: 66.1%, mp: 210–212 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.83 (s, 1H, 5-OH), 7.41–7.38 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.56 (s, 1H, aromatic H_8), 6.48 (s, 1H, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.75 (s, 2H, –CH_2O–), 4.71 (s, 2H, –CH_2ONO_2), 4.33(d, 2H, J = 3.9 Hz, –CH_2OCO–). MALDI-TOF: m/z 460 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{17}NO_{11}$: C, 54.91; H, 3.73; N, 3.05. Found: C, 54.53; H, 3.58; N, 2.93.

*Compound **Lb3***: Compound **5b** (253 mg, 0.5 mmol), $AgNO_3$ (0.85 g, 5 mmol), yield: 53.3%, mp: 143–145 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 12.83 (s, 1H, 5-OH), 7.41–7.38 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.56 (s, 1H, aromatic H_8), 6.48 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.71 (s, 2H, –CH_2O–), 4.46 (s, 2H, –CH_2ONO_2), 4.34–4.33 (d, 4H, J = 4 Hz, –CH_2CH_2–), 4.27 (s, 2H, –CH_2OCO–), 1.80 (s, 4H, –CH_2CH_2–). MALDI-TOF: m/z 488 ([M+H]$^+$). Anal. Calcd for $C_{23}H_{21}NO_{11}$: C, 56.68; H, 4.34; N, 2.87. Found: C, 56.52; H, 4.18; N, 2.73.

*Compound **Lb5***: Compound **5c** (267 mg, 0.5 mmol), $AgNO_3$ (0.85 g, 5 mmol), yield: 68.1%, mp: 138–140 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 12.82 (s, 1H, 5-OH), 7.41–7.37 (m, 2H, aromatic $H_{2',6'}$), 6.99–6.97 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.56 (s, 1H, aromatic H_8), 6.48 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.70 (s, 2H, –CH_2O–), 4.43–4.40 (t, 2H, J = 6.4 Hz, –CH_2ONO_2), 4.33 (d, 4H, J = 3.6 Hz, –CH_2CH_2–), 4.25–4.21 (t, 2H, J = 6.3 Hz, –CH_2OCO–), 1.72–1.67 (q, 4H, J = 6.5 Hz, CH_2, CH_2), 1.40 (s, 4H, –CH_2CH_2–). MALDI-TOF: m/z 516 ([M+H]$^+$). Anal. Calcd for $C_{25}H_{25}NO_{11}$: C, 58.25; H, 4.89; N, 2.72. Found: C, 57.96; H, 4.68; N, 2.53.

4.2.4. General procedures for the synthesis of compounds Lc1–6 and 8a–c

4.2.4.1. O3′,O4′-Diphenylmethane luteolin (6). Dichlorodiphenylmethane (2 mL, 10.42 mmol) was added to a stirred mixture of luteolin (2 g, 6.99 mmol) in diphenyl ether (20 mL) and the reaction mixture was heated at 175 °C for 30 min.

After cooled to 60 °C, the dark solution was poured into petroleum (100 mL), the precipitation was filtered. The filtrate was concentrated and purified by column chromatography (eluent, petroleum ether/EtOAc = 6:1 to 2:1) to give **6**, respectively. Yield: 50.7%, yellow solid, mp: 139–141 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 12.86 (s, 1H, 5-OH), 10.86 (s, 1H, 7-OH), 7.78 (s, 1H,

aromatic $H_{2'}$), 7.72–7.70 (d, 1H, J = 8 Hz, aromatic $H_{6'}$), 7.56–7.46 (m, 10H, diphenylmethylene H), 7.24–7.22 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.88 (s, 1H, aromatic H_8), 6.52 (s, 1H, aromatic H_3), 6.20 (s, 1H, aromatic H_6).

4.2.4.2 O7-Bromethyl-O3′,O4′-diphenylmethane luteolin (**7a–c**). To a stirred solution of **6** (1.8 g, 4 mmol) in acetone (150 mL), was added anhydrous potassium carbonate (0.6 g, 4.4 mmol), the reaction mixture was stirred at reflux for 30 min. 1,2-Dibromoethane (12 mmol, 1.1 mL) was then added dropwise for 10 min. The mixture was stirred at reflux for 24 h, the precipitation was filtered. The filter liquor was concentrated under reduced pressure to obtain a yellow solid. The solid was washed with water (3 × 20 mL), dried and purified by column chromatography (eluent, CH_2Cl_2) to give **7a**, respectively. Yield: 70.3%, yellow solid, mp: 127–129 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.78 (s, 1H, 5-OH), 7.58–7.57 (m, 4H, aromatic $H_{2',6'}$, aromatic H, aromatic H), 7.47–7.39 (m, 8H, diphenylmethylene H), 7.00–6.98 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.53 (s, 1H, aromatic H_8), 6.47 (s, 1H, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.36–4.33 (t, 2H, J = 6 Hz, –CH_2O–), 3.67–3.64 (t, 2H, J = 6 Hz, –CH_2Br).

Compounds **7b** and **7c** were synthesized according to the method for **7a**.

Compound **7b**: 1,4-Dibromobutane (12 mmol, 1.5 mL), yield: 69.8%, yellow solid, mp: 150–152 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.74 (s, 1H, 5-OH), 7.58–7.57 (m, 4H, aromatic $H_{2',6'}$, aromatic H, aromatic H), 7.46–7.39 (m, 8H, diphenylmethylene H), 7.00–6.98 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.52 (s, 1H, aromatic H_8), 6.44 (s, 1H, aromatic H_3), 6.33 (s, 1H, aromatic H_6), 4.08–4.05 (t, 2H, J = 5.2 Hz, –CH_2O–), 3.51–3.48 (t, 2H, J = 6.1 Hz, –CH_2Br), 2.09–1.98 (m, 4H, –CH_2CH_2–).

Compound **7c**: 1,6-Dibromohexane (12 mmol, 1.9 mL), yield: 83.1%, yellow solid, mp: 110–112 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.74 (s, 1H, 5-OH), 7.58–7.57 (m, 4H, aromatic $H_{2',6'}$, aromatic H, aromatic H), 7.47–7.39 (m, 8H, diphenylmethylene H), 7.00–6.98 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.52 (s, 1H, aromatic H_8), 6.44 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.04–4.01 (t, 2H, J = 5.6 Hz, –CH_2O–), 3.45–3.42 (t, 2H, J = 6.4 Hz, –CH_2Br), 1.91–1.83 (m, 4H, CH_2, CH_2), 1.52 (s, 4H, –CH_2CH_2–).

4.2.4.3. O7-Nitrooxyethyl-O3′,O4′-diphenylmethane luteolin (8a–c). A solution of the appropriate bromo derivatives **7a–c** (2 mmol) and $AgNO_3$ (3 g, 20 mmol) in dry CH_3CN (60 mL) and THF (20 mL) was stirred at 75 °C. The reaction was allowed to proceed for 8–12 h in the dark. Filtration was performed to remove any silver bromide precipitates. The filtrate was concentrated under reduced pressure and a yellow solid was produced. This solid was washed with water until the runoff was clear. Solid samples were dried and purified by column chromatography (eluent, petroleum ether/EtOAc = 8:1 to CH_2Cl_2) to afford **8a–c**, respectively.

Compound **8a**: Compound **7a** (1.12 g, 2 mmol), yield: 73.9%, yellow solid, mp: 98–100 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.81 (s, 1H, 5-OH), 7.58 (s, 4H, aromatic $H_{2',6'}$, aromatic H, aromatic H), 7.47–7.40 (m, 8H, diphenylmethylene H), 7.01–6.99 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.54 (s, 1H, aromatic H_8), 6.46 (s, 1H, aromatic H_3), 6.34 (s, 1H, aromatic H_6), 4.85 (s, 2H, –CH_2ONO_2), 4.31 (s, 2H, –CH_2O–).

Compound **8b**: Compound **7b** (1.17 g, 2 mmol), yield: 71.9%, yellow solid, mp: 129–131 °C. 1H NMR (400 MHz, $CDCl_3$) δ: 12.77 (s, 1H, 5-OH), 7.58 (s, 4H, aromatic $H_{2',6'}$, aromatic H, aromatic H), 7.47–7.40 (m, 8H, diphenylmethylene H), 7.00–6.99 (d, 1H, J = 7.2 Hz, aromatic $H_{5'}$), 6.53 (s, 1H, aromatic H_8), 6.44 (s, 1H, aromatic H_3), 6.33 (s, 1H, aromatic H_6), 4.55 (s, 2H, –CH_2ONO_2), 4.07 (s, 2H, –CH_2O–), 1.83 (s, 4H, –CH_2CH_2–).

Compound **8c**: **7c** (1.23 g, 2 mmol), yield: 76.3%, yellow solid, mp: 153–155 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.75 (s, 1H, 5-OH), 7.58–7.57 (m, 4H, aromatic $H_{2',6'}$, aromatic H, aromatic H), 7.47–7.39 (m, 8H, diphenylmethylene H), 7.00–6.98 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.52 (s, 1H, aromatic H_8), 6.44 (s, 1H, aromatic H_3), 6.33 (s, 1H, aromatic H_6), 4.48–4.45 (t, 2H, J = 6.1 Hz, –CH_2ONO_2), 4.02 (s, 2H, –CH_2O–), 1.83–1.77 (m, 4H, CH_2, CH_2), 1.51 (s, 4H, –CH_2CH_2–).

4.2.4.4. O7-Nitrooxyethyl luteolin (Lc1, Lc3, Lc5). Compounds **8a–c** (1 mmol) was added to a mixture of acetic acid/water (80:20, 50 mL). The solution was refluxed for 4–5 h. Then EtOAc (50 mL) and water (50 mL) were added. The organic layer was washed with a $NaHCO_3$ saturated aqueous solution (3 × 30 ml) and dried over anhydrous $MgSO_4$. After the solution was concentrated, the residue was purified by recrystallization from CH_2Cl_2 to afford **Lc1**, **Lc3**, **Lc5**, respectively.

Compound **Lc1**: Compound **8a** (539.5 mg, 1 mmol), yield: 45.5%, yellow solid, mp: 228–230 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 13.00 (s, 1H, 5-OH), 10.02 (s, 1H, 4′-OH), 9.39 (s, 1H, 3′-OH), 7.46–7.43 (m, 2H, aromatic $H_{2',6'}$), 6.91–6.89 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.79–6.75 (d, 2H, J = 13.3 Hz, aromatic H_8, aromatic H_3), 6.41 (s, 1H, aromatic H_6), 4.91 (s, 2H, –CH_2ONO_2), 4.46 (s, 2H, –CH_2O–). MALDI-TOF: m/z 376 ([M+H]$^+$). Anal. Calcd for $C_{17}H_{13}NO_9$: C, 54.41; H, 3.49; N, 3.73. Found: C, 54.12; H, 3.28; N, 3.51.

Compound **Lc3**: Compound **8b** (567.5 mg, 1 mmol), yield: 43.7%, yellow solid, mp: 224–226 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 12.98 (s, 1H, 5-OH), 10.00 (s, 1H, 4′-OH), 9.39 (s, 1H, 3′-OH), 7.43 (s, 2H, aromatic $H_{2',6'}$), 6.90–6.89 (d, 1H, J = 6.4 Hz, aromatic $H_{5'}$), 6.73 (s, 2H, ArH_8, aromatic H_3), 6.37 (s, 1H, aromatic H_6), 4.60 (s, 2H, –CH_2ONO_2), 4.15 (s, 2H, –CH_2O–), 1.83 (s, 4H, –CH_2CH_2–). MALDI-TOF: m/z 488 ([M+H]$^+$). Anal. Calcd for $C_{19}H_{17}NO_9$: C, 56.58; H, 4.25; N, 3.47. Found: C, 56.29; H, 4.02; N, 3.12.

Compound **Lc5**: Compound **8c** (595.6 mg, 1 mmol), yield: 54.5%, yellow solid, mp: 203–205 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 12.96 (s, 1H, 5-OH), 9.98 (s, 1H, 4′-OH), 9.37 (s, 1H, 3′-OH), 7.45–7.43 (m, 2H, aromatic $H_{2',6'}$), 6.90–6.88 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.72 (s, 2H, aromatic H_8, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.54–4.51 (t, 2H, J = 6.4 Hz, –CH_2ONO_2), 4.11–4.08 (t, 2H, J = 6 Hz, –CH_2O–), 1.75–1.68 (m, 4H, CH_2, CH_2), 1.43 (s, 4H, –CH_2CH_2–). MALDI-TOF: m/z 432 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{21}NO_9$: C, 58.47; H, 4.91; N, 3.25. Found: C, 58.16; H, 4.69; N, 2.97.

4.2.4.5. O7-Ethyl luteolin (Lc2, Lc4, Lc6). Bromoethane (0.38 mL, 5 mmol) was added to a stirred solution of luteolin (2.86 g, 10 mmol) and anhydrous potassium carbonate (0.7 g, 5 mmol) in dry DMF (50 mL) and the reaction mixture was heated at 70 °C for 3 h.The mixture was added ice water, dropwise. The precipitation was filtered, washed with water, dried under reduced pressure. The residue was purified by column chromatography (eluent, CH_2Cl_2/EtOAc = 20:3 to 2:1) to afford **Lc2**, respectively. Yield: 7.85%, yellow solid, mp: 230–232 °C. 1H NMR (400 MHz, DMSO-d_6, ppm) δ: 12.97 (s, 1H, 5-OH), 9.98 (s, 1H, 4′-OH), 9.39 (s, 1H, 3′-OH), 7.45–7.43 (m, 2H, aromatic $H_{2',6'}$), 6.90–6.88 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.73–6.70 (d, 2H, J = 10.1 Hz, aromatic H_8, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.18–4.13 (q, 2H, J = 6.7 Hz, –CH_2O–), 1.37–1.34 (t, 3H, J = 6.8 Hz, –CH_3). MALDI-TOF: m/z 315 ([M+H]$^+$).

Compounds **Lc4** and **Lc6** were synthesized according to the method for **Lc2**.

Compound **Lc4**: Luteolin (2.86 g, 10 mmol), bromobutane (0.54 mL, 5 mmol), yield: 7.63%, yellow solid, mp: 218–220 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 12.96 (s, 1H, 5-OH), 9.98 (s, 1H, 4′-OH), 9.37 (s, 1H, 3′-OH), 7.46–7.43 (m, 2H, aromatic $H_{2',6'}$), 6.90–6.88 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.73 (s, 2H, aromatic H_8,

aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.11–4.08 (t, 2H, J = 5.9 Hz, $-CH_2O-$), 1.74–1.70 (t, 2H, J = 6.7 Hz, $-CH_2-$), 1.47–1.41 (q, 2H, J = 7.2 Hz, $-CH_2-$), 0.96–0.92 (t, 3H, J = 7.2 Hz, $-CH_3$). MALDI-TOF: m/z 343 ($[M+H]^+$).

Compound **Lc6**: Luteolin (2.86 g, 10 mmol), bromohexane (0.71 mL, 5 mmol), yield: 8.57%, yellow solid, mp: 243–245 °C. 1H NMR (400 MHz, DMSO-d_6, ppm) δ: 12.96 (s, 1H, 5-OH), 9.99 (s, 1H, 4′-OH), 9.37 (s, 1H, 3′-OH), 7.46–7.43 (m, 2H, aromatic $H_{2',6'}$), 6.90–6.88 (d, 1H, J = 8 Hz, aromatic $H_{5'}$), 6.72 (s, 2H, aromatic H_8, aromatic H_3), 6.35 (s, 1H, aromatic H_6), 4.09–4.07 (d, 2H, J = 5.8 Hz, $-CH_2O-$), 1.73 (s, 2H, $-CH_2-$), 1.41–1.31 (m, 6H, $-CH_2CH_2CH_2-$), 0.88 (s, 3H, $-CH_3$). MALDI-TOF: m/z 371 ($[M+H]^+$).

4.3. Biological activity

4.3.1. Detection of nitric oxide[24]

A solution of the appropriate compound (20 μL) in dimethylsulfoxide (DMSO) was added to 2 mL of 1:1 v/v mixture of 50 mM PBS (pH 7.4) with MeOH, containing of 5×10^{-4} M L-cysteine.

The final concentration of target compounds was 10^{-4} M. After 1 h at 37 °C, 1 mL of the reaction mixture was treated with 250 μL of Griess reagent [sulfanilamide (4 g), *N*-naphthylethylenediamine dihydrochloride (0.2 g), 85% phosphoric acid (10 mL) in distilled water (final volume: 100 mL)]. After 10 min at room temperature, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–80 nmol/mL) were used to construct the calibration curve. The results were expressed as the percentage of NO released (n = 3) relative to a theoretical maximum release of 1 mol NO/mol of test compound.

4.3.2. Preparation of aldose reductase[31,32]

Calf eyes were obtained from a local abattoir soon after slaughtering. Lenses were removed and kept frozen at −20 °C until use. Purified calf eye extract was prepared by ammonium sulphate fractionation. The extract that showed AR activity was redissolved in 5 M PBS and dialysed overnight in the same solution. The dialysed material was used for enzymatic assay and stored at −70 °C.

4.3.3. Enzymatic inhibition[24,32,33]

Test enzyme and inhibitory activities of the target compounds were determined spectrophotometrically by monitoring changes in absorbance at 340 nm. Such changes are due to the oxidation of NADPH catalysed by AR.

Determination of the AR inhibitory activities of the newly synthesised compounds was conducted using an optimised volume of 200 μL of enzyme (750 μg/mL protein) and different concentrations of the compounds (20 μL, 0.1–10 μmol/L) in 50 mM PBS (pH 6.2) containing β-mercaptoethanol (20 μL, 5 mM), NADPH (20 μL, 0.24 mM), Li_2SO_4 (40 μL, 0.4 M), and glyceraldehyde (20 μL, 2.5 mM). The reaction was initiated by addition of glyceraldehyde, and the decrease in optical density of NADPH at 340 nm was recorded for 10 min. The IC_{50} values of the compounds were calculated using Sigmaplot software and expressed as the mean ± S.D. of triplicate experiments. The flavonoid quercetin was used as a reference during the AR assay.

4.3.4. Statistical analysis

Data were shown as mean ± S.D. Differences between individual groups were analyzed by using ANOVA followed by Dunett's test. A difference with a P value of <0.05 was considered to be significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.04.066.

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