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ARTICLE

Synthesis and biological evaluation of flavonol-glucose conjugates for cosmeceutical development

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Abstract Quercetin and kaempferol, two well-known flavonols, were chemically conjugated with glucose to produce the corresponding flavonol glucosides, and the following biological activities were evaluated for cosmeceutical development: antioxidant activity, ability to increase collagen synthesis, and moisturizing activity. Among the synthetic flavonol glucosides, quercetin-3-*O*-β-D-glucoside significantly enhanced collagen synthesis (60 %) compared to quercetin. Kaempferol-3,7-di-*O*-β-D-glucoside showed promising skin-moisturizing effects, inducing a sixfold increase in the expression of aquaporin-3. Thus, both quercetin-3-*O*-β-D-glucoside and kaempferol-3,7-di-*O*-β-D-glucoside were shown to possess interesting biological activities which warrant their further development as cosmetic ingredients.

Keywords Cosmetic ingredient · Kaempferol glucoside · Quercetin glucoside · Collagen synthesis · Aquaporin-3

Introduction

Many flavonoids, due to their various biological activities, are used in pharmaceutical, cosmetic, and food preparations (Ardhaoui et al. 2004; Ranger et al. 2007; Birbara, 2011). Quercetin and kaempferol are natural flavonols (Vuorinen et al. 2000; Hashimoto et al. 2006), abundant in fruit, vegetables, and tea. Their various biological effects, including anticancer, antioxidant, and anti-inflammatory activities, have been the focus of several studies (Pang et al. 2006; Lee et al. 2010; Nirmala and Ramanathan 2011). Quercetin and kaempferol also possess anti-aging (Niki et al. 2010), whitening (Kubo et al. 2007), and anti-wrinkle activity (Chuarienthong et al. 2010), which supports their potential for use as cosmetic ingredients. However, their usage is often limited due to suboptimal biological activity, as well as unfavorable pharmacokinetic properties such as low solubility, low absorption, and rapid metabolism (Li et al. 2012). Therefore, the potential use of quercetin and kaempferol as cosmetic ingredients has not been well elucidated, and ultimately only a limited number of plant extracts containing these flavonoids have been utilized for cosmetic purposes (dal Belo et al. 2009). It is worth noting that most flavonoids obtained from plant sources are in the form of *O*-glycosides, whose bioactivity as well as bioavailability depends on the type and position of the conjugated sugar (Chang et al. 2005). In particular, a glucose functionality attached to quercetin was shown to significantly enhance its bioavailability, irrespective of attachment position (Murota et al. 2004; Walgren et al. 2000). Thus, the conjugation of flavonols with glucose is a promising strategy to potentiate biological activity and optimize bioavailability. Nevertheless, it is not easy to obtain flavonoid glucosides from natural sources on a large-scale basis because they are present in low concentrations in plant sources and can undergo facile hydrolysis upon extraction (Zhang et al. 2014).

Quercetin-3-*O*-β-D-glucoside and kaempferol-3,7-di-*O*-β-D-glucoside were chemically synthesized in this study, and the following biological activities were screened for potential cosmetic use: antioxidant activity, ability to stimulate collagen synthesis, and moisturizing activity.

Kwang-Su Park, Hyungmi Kim, have contributed equally to this work.

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Antioxidant effect was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and collagen synthesis activity was quantified by the amount of procollagen type I C-peptide produced. The moisturizing effect was estimated using the mRNA level of aquaporin-3, the most important water channel in the skin. (Fig. 1).

Materials and methods

Materials

All chemical reagents, including DPPH, quercetin, and kaempferol, were purchased from Sigma-Aldrich, TCI, or Alfa. Dulbecco's modified eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen. The procollagen type I C-peptide ELISA kit was manufactured by TAKARA. The quick RT-PCR kit to detect aquaporin mRNA levels and the RNA extraction kit were purchased from QIAGEN. Nuclear magnetic resonance spectra were recorded on a Bruker 400 AMX spectrometer (Karlsruhe, Germany) at 400 and 100 MHz for ^{1}H and ^{13}C NMR respectively, with tetramethylsilane as an internal standard. UV absorbance was recorded using a SpectraMax M2e (Molecular Devices, USA).

Chemistry

Quercetin-3-O-β-D-glucoside (3)

Quercetin **1** (7.5 g, 22.2 mmol) and (2*R*,3*R*,4*S*,5*R*,6*R*)-2-(acetoxymethyl)-6-bromotetrahydro-2*H*-pyran-3,4,5-triyl triacetate (8.2 g, 19.9 mmol) were dissolved in 1,4-dioxane (225 mL). K_2CO_3 (15.3 g, 110.8 mmol) was added to the solution. The resulting mixture was stirred for 8 h at 100 °C. After cooling to room temperature, the reaction mixture was filtered by filter paper, and the filtrate was concentrated under reduced pressure to afford quercetin-3-*O*-β-D-peracetylglucoside, which was used for the next reaction without further purification. The compound was dissolved in MeOH (400 mL) and treated with 25 % NaOMe in MeOH (3 mL). The resulting mixture was stirred for 30 min at room temperature, and then an excess amount of H_2O was poured into the reaction mixture. After the mixture was passed through an cationic exchange resin (Fluka Amberlite IR-120 H^+ form 16–45 mesh), the resulting solution was concentrated under reduced pressure to give the desired quercetin glucoside **3** (7.1 g, 15.3 mmol, 68 % yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.70 (d, $J = 2.0$ Hz, 1H), 7.57 (d, $J = 8.6$ Hz, 1H), 6.75 (dd, $J = 8.6$, 2.0 Hz, 1H), 6.33 (d, $J = 2.0$ Hz, 1H), 6.15 (d, $J = 2.0$ Hz, 1H), 5.23 (d, $J = 7.7$ Hz, 1H), 3.18–3.78 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 62.5, 71.2, 75.7, 78.1, 78.3, 94.7, 99.9, 104.3, 105.7, 116.0, 117.6, 123.0, 123.2, 135.6, 145.7, 149.8, 158.4, 159.0, 162.9, 165.9, 179.4.

3,5-Bis(benzyloxy)-2-(2-(benzyloxy)acetyl)phenyl 4-(methoxymethoxy)benzoate (6)

Compound **5** (2.0 g, 4.4 mmol) and 4-(methoxymethoxy)-benzoic acid (0.8 g, 4.4 mmol) were dissolved in *N*,*N*-dimethylformamide (DMF, 5 mL). 4-Dimethylaminopyridine (DMAP, 0.5 g, 4.4 mmol) and 1-ethyl-3-(3-

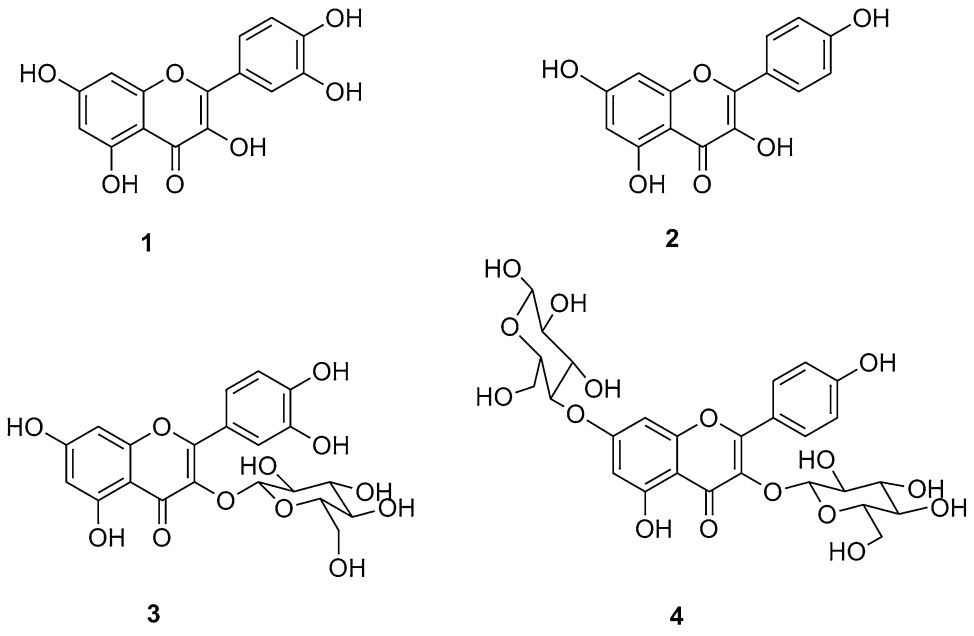


Fig. 1 Structures of quercetin (**1**), kaempferol (**2**), quercetin-3-*O*-β-D-glucoside (**3**), and kaempferol-3,7-di-*O*-β-D-glucoside (**4**)

dimethylaminopropyl) carbodiimide (EDC, 2.1 g, 13.2 mmol) were added to this solution, and the resulting mixture was stirred for 4 h at room temperature. After the volatile compounds were evaporated under reduced pressure, the residue was purified by column chromatography on silica gel (5:1 = Hex:EtOAc) to give the desired product **6** (2.2 g, 3.6 mmol, 81 % yield) as a pale yellow solid: ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.06 (d, J = 8.9 Hz, 2H), 7.50 (d, J = 8.9 Hz, 4H), 7.35-7.43 (m, 6H), 7.17-7.27 (m, 7H), 6.80 (d, J = 2.1 Hz, 1H), 6.67 (d, J = 2.1 Hz, 1H), 5.32 (s, 2H), 5.23 (s, 2H), 5.19 (s, 2H), 4.51 (s, 2H), 4.40 (s, 2H), 3.46 (s, 3H).

3,7-Di-O-benzyl-4′-O-methoxymethyl kaempferol (7)

Compound **6** (2.2 g, 3.6 mmol) was dissolved in toluene (10 mL). K_2CO_3 (1.8 g, 14.4 mmol) and tetrabutylammonium bromide (TBAB, 1.0 g, 3.2 mmol) were added, and the resulting mixture was stirred for 4 h at 90 °C. After cooling to room temperature, the mixture was filtered, and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (4:1 = Hex:Acetone) to afford the desired product **7** (1.5 g, 2.5 mmol, 69 % yield) as a yellow powder: ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.80 (s, 1H), 7.97 (d, J = 9.0 Hz, 2H), 7.28-7.45 (m, 10H), 7.09 (d, J = 9.0 Hz, 2H), 6.50 (d, J = 2.2 Hz, 1H), 6.44 (d, J = 2.2 Hz, 1H), 5.24 (s, 2H), 5.13 (s, 2H), 5.07 (s, 2H), 3.51 (s, 3H).

4′-O-Methoxymethyl kaempferol (8)

Compound **7** (1.0 g, 2.0 mmol) was dissolved in a mixture of MeOH (2 mL) and tetrahydrofuran (THF, 4 mL). Pd/C (10 % w/w, 0.2 mmol) was added to this solution, and the resulting mixture was stirred for 4 h under a H_2 atmosphere (balloon) at room temperature. The mixture was filtered through a Celite pad and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (2:1 = Hex:Acetone) to afford 4′-O-methoxymethyl kaempferol **8** (0.6 g, 1.7 mmol, 85 % yield) as a yellow powder: ^{1}H NMR (400 MHz, Acetone-d_6) δ 12.80 (s, 1H), 8.15 (d, J = 8.8 Hz, 2H), 7.09 (d, J = 8.8 Hz, 2H), 6.74 (d, J = 2.1 Hz, 1H), 6.43 (d, J = 2.0 Hz, 1H), 5.30 (s, 4H), 3.45 (s, 6H).

Kaempferol-3,7-di-O-β-D-glucoside (4)

Compound **8** (1.0 g, 3.0 mmol) was dissolved in a mixture of $CHCl_3$ (30 mL) and H_2O (30 mL). Peracetylated 1-bromoglucose (3.7 g, 9.1 mmol) and Aliquat 336 (0.6 mL, 1.3 mmol) were added, and the resulting mixture was stirred for 18 h at 50 °C. After cooling to room temperature, the mixture was washed with 1 N HCl, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (2:1 = Hex:EtOAC) to give the protected diglucoside (1.6 g, 1.6 mmol), which was then dissolved in CH_2Cl_2 (15 mL). Trifluoroacetic acid (TFA, 1.5 mL, 19.3 mmol) was added to this solution at 0 °C, before stirring for 4 h at room temperature. The mixture was then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (1:1 = Hex:Acetone) to give the 4′-O-MOM deprotected product (0.9 g, 0.9 mmol), which was dissolved in methanolic ammonia (20 mL) at 0 °C. The resulting mixture was stirred for 1 h at room temperature and then concentrated under reduced pressure to afford the kaempferol diglucoside **4** (0.5 g, 0.8 mmol, 85 % yield) as a yellow powder: ^{1}H NMR (400 MHz, MeOD) δ (ppm) 8.08 (d, J = 8.8 Hz, 2H), 6.88 (d, J = 8.9 Hz, 2H), 6.77 (d, J = 2.1 Hz, 1H), 6.49 (d, J = 2.1 Hz, 1H), 5.32 (d, J = 7.4 Hz, 1H), 5.06 (d, J = 7.2 Hz, 1H), 3.92 (dd, J = 12.2, 2.1 Hz, 1H), 3.71 (dd, J = 12.1, 5.6 Hz, 1H), 3.38-3.56 (m, 9H), 3.19–3.23 (m, 1H).

DPPH radical scavenging assay

Scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined as follows: An ethanol solution of DPPH (10 μM) was incubated with the test compounds at various concentrations. After a 1 h incubation at room temperature, absorbance was recorded at 517 nm using a microplate reader. Vitamin C was used as a positive control.

Quantification of procollagen type I C-peptide

Collagen amounts were detected using the procollagen type I C-peptide (PIP) ELISA kit (TAKARA, Japan) according to the manufacturer's instructions. Briefly, human fibroblast CCD986sk cells (1×10^6) were seeded in a 6-well plate at 37 °C. After 24 h, the synthesized test compounds (10 μg/ml) were added, and the plate was incubated for 48 h at 37 °C. The media from the 6-well plate were collected to detect the amount of procollagens secreted into the media. The HRP-conjugated anti-PIP monoclonal antibody solution (100 μL) was added to the anti-PIP monoclonal antibody-precoated microplate from the kit. Then, 20 μL of the sample media was added to the plate. The plate was wrapped with foil and incubated for 3 h at 37 °C. After incubation, the solution was removed and the plate was washed 4 times with 400 μL washing buffer. Substrate solution (100 μL) was added to the plate followed by a 15 min incubation at room temperature. Finally, 100 μL of stop solution was added to the microplate, and the absorbance was recorded by UV spectrophotometer at 450 nm.

Estimation of mRNA level of aquaporin-3

The mRNA level of aquaporin-3 was monitored by RT-PCR using the QuantiTect SYBR Green PCR kit (QIAGEN). HaCaT cells (1×10^5) were plated in a 24-well microplate. After incubation for 24 h, the synthesized test compounds (10 μg/ml) were added to the cells, which were then incubated for 72 h at 37 °C. Total RNA was extracted using the RNeasy-Mini-kit (QIAGEN) according to the manufacturer's instructions. The prepared RNA was used as a template for RT-PCR using the primer of aquaporin-3. RT-PCR was performed by QuantiTect SYBR green PCR kit according to the manufacturer's instructions. The result was normalized by GAPDH using a GAPDH primer.

Results and discussion

Synthesis of quercetin-3-*O*-β-D-glucoside (3) and kaempferol-3,7-di-*O*-β-D-glucoside (4)

Novel methods for the preparation of both quercetin-3-*O*-β-D-glucoside (**3**) and kaempferol-3,7-di-*O*-β-D-glucoside (**4**) were developed; these methods could be applied to large-scale synthesis. Synthesis of quercetin-3-*O*-β-D-glucoside was accomplished in two steps beginning from quercetin (**1**) (Scheme 1). In general, due to the presence of five hydroxyl groups, regioselective glycosylation of quercetin remains a challenge. However, we managed to optimize the process and produce a high yield using 1,4-dioxane as a solvent. Peracetyl 1-bromoglucose was obtained via treatment of peracetylated glucose with HBr, and then reacted with quercetin (**1**) in the presence of K_2CO_3 in 1,4-dioxane to give 3-*O*-glucosyl quercetin. The resulting product was deacetylated using methanolic ammonia to produce quercetin-3-*O*-glucose **3** with a 68 % yield (Scheme 1).

Regioselective introduction of glucose at both the 3 and 7 positions of kaempferol required additional steps (Scheme 2). Due to the high cost as well as lack of regioselectivity, it was not feasible to prepare the desired kaempferol glucoside by direct glycosylation. Thus, to synthesize kaempferol-3,7-di-*O*-β-D-glucoside (**4**), a 4′-*O*-protected kaempferol scaffold (**8**) was prepared from quercetin. Compound **5,** obtained by fragmentation of perbenzylated quercetin (Caldwell et al. 2006), was coupled with 4′-methoxymethoxybenzoic acid under EDC coupling conditions to give the intermediate **6** with an 81 % yield. The intermediate was then cyclized in the presence of K_2CO_3 and TBAB in toluene to give the protected kaempferol **7** with a 69 % yield. Debenzylation of **7** produced compound **8** which was conjugated with peracetylated 1-bromoglucose. After removal of the 4′-*O*-MOM protecting group under acidic conditions, deacetylation was achieved using methanolic ammonia to give the kaempferol-3,7-di-*O*-β-D-glucoside **4** with an 87 % yield.

Evaluation of biological activity for cosmetic use

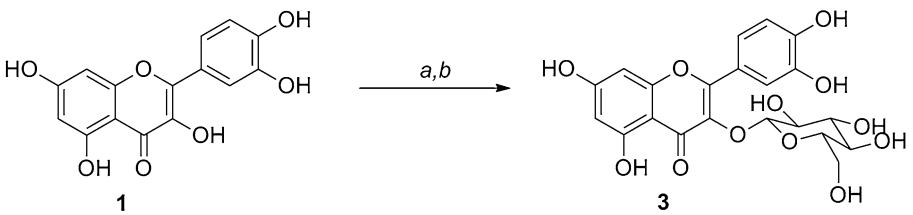
Antioxidant effect of flavonols and their glucose conjugates

The antioxidant effect of the flavonol aglycones (**1** and **2**) and their glucose conjugates (**3** and **4**) were evaluated by a DPPH assay at various concentrations. Excluding kaempferol-3,7-di-*O*-β-D-glucoside (**4**), the flavonols (**1** ∼ **3**) showed moderate antioxidant activity at concentrations ≥10 μg/mL (Fig. 2). Quercetin (**1**) showed the most potent antioxidant effect, presumably due to its free catechol moiety. In contrast, quercetin-3-*O*-β-D-glucoside (**3**) had a reduced antioxidant effect compared to quercetin (**1**). Blocking the free phenolic group with glucose seemed to prevent the compound from reacting with the free radical. In case of kaempferol, the aglycone form (**2**) was also more effective than the corresponding diglucoside (**4**). This suggested that the single-free phenolic hydroxyl group of kaempferol-3,7-di-*O*-β-D-glucoside could not participate in radical scavenging. Overall, the flavonol aglycones demonstrated greater radical scavenging ability than the corresponding flavonol glucosides.

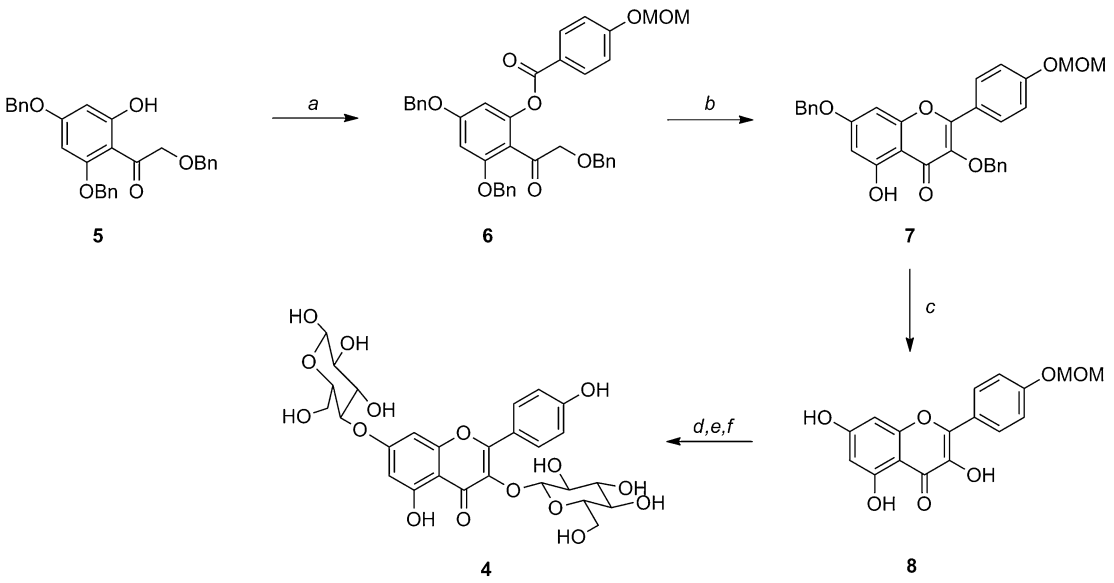
Effect of the flavonols and their glucose conjugates on collagen synthesis

Collagen is the most abundant protein in the skin and is primarily composed of type I (80 %) and type III (10–15 %) collagen peptides. In the human skin cell,

Scheme 1 Synthesis of quercetin-3-*O*-β-D-glucoside (**3**)



Reagents and Conditions: (a) peracetylated 1-bromoglucose, K_2CO_3,1,4-dioxane, reflux; (b) NH_3/MeOH



Reagents and Conditions: a) 4-(Methoxymethyl)benzoic acid, EDC, DMAP, CH_2Cl_2, rt ; b) TBAB, K_2CO_3, tol. 90 °C; c) H_2, Pd/C, MeOH; d) peracetylated 1-bromoglucose, $CHCl_3/H_2O$, K_2CO_3, Aliquat 336, 50 °C; e) TFA, CH_2Cl_2, 0 °C to rt; f) NH_3, MeOH

Scheme 2 Synthesis of kaempferol-3,7-di-*O*-β-D-glucoside (**4**)

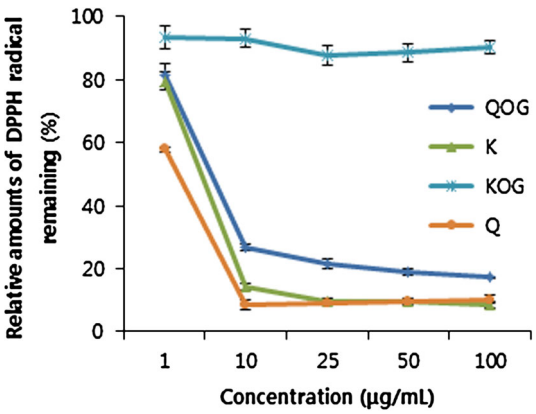


Fig. 2 Antioxidant activity of various concentrations of flavonols. Q, quercetin (**1**); K, kaempferol (**2**); QOG, quercetin-3-*O*-β-D-glucoside (**3**); KOG, kaempferol-3,7-di-*O*-β-D-glucoside (**4**)

collagen has an important role in morphogenesis and growth (Haapasaari et al. 1998). The effects of the flavonols on collagen synthesis were evaluated by measuring the amount of type I collagen, the dominant component in collagen, using the collagen type I C-peptide ELISA kit. All compounds tested (10 μg/ml) induced an increase in collagen synthesis compared with the control (only vehicle treated). In the case of quercetin (**1**), collagen synthesis was only increased by 10 %, while quercetin-3-*O*-β-D-glucoside (**3**) showed a 60 % enhancement. Kaempferol (**2**) was more effective (30 % induction) than kaempferol-3,7-di-*O*-β-D-glucoside (**4**) (10 % induction) (Fig. 3). Overall, quercetin-3-*O*-β-D-glucoside (**3**) was the most effective compound, exhibiting a 60 % increase in collagen synthesis.

Moisturizing effect of flavonols and their glucose conjugates

Aquaporin-3 (AQP3) is a membrane transporter that functions in delivering water and glycerol. There are 13 kinds of AQPs (AQP0-AQP12) in mammals. Among them, AQP3 is abundant in skin, and it has an important role in skin hydration (Boury-Jamot et al. 2006). Thus, the AQP3 expression level is closely linked to skin moisturization. The flavonols and their glucose conjugates (10 μg/ml) were evaluated for their moisturizing effect using the QuantiTect SYBR Green PCR kit which performs quantitative RT-PCR to monitor the mRNA expression level of AQP3. Interestingly, all of the tested compounds showed an enhancement of mRNA expression compared with the control. Compared with the vehicle control treatment, kaempferol-3,7-di-*O*-β-D-glucoside (**4**) increased AQP3 expression (sixfold) more than kaempferol aglycone (**2**) (twofold) did. On the other hand, compared with control, quercetin-3-*O*-β-D-glucoside (**3**) increased AQP3 expression (twofold) less than quercetin aglycone (**1**) (fourfold) did (Fig. 4). Overall, the kaempferol glucoside-treated cells showed the highest expression of AQP3, but both flavonols and their glucose conjugates demonstrated some activity when compared to the control.

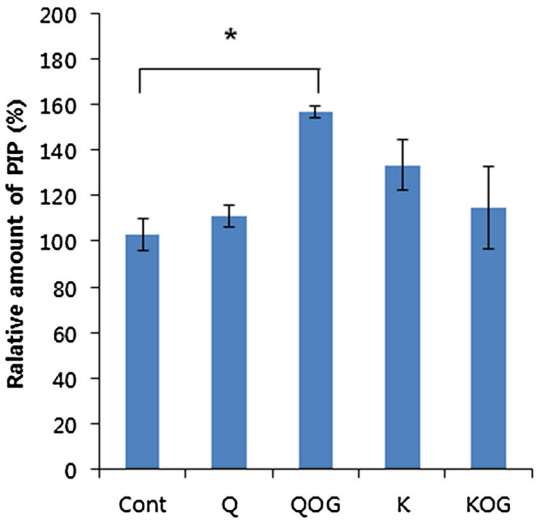


Fig. 3 Effects of flavonols (10 μg/ml) on collagen synthesis in CCD986sk cells as determined by sandwiched ELISA. Q, quercetin (**1**); K, kaempferol (**2**); QOG, quercetin-3-*O*-β-D-glucoside (**3**); KOG, kaempferol-3,7-di-*O*-β-D-glucoside (**4**); Cont, DMSO. *p < 0.01

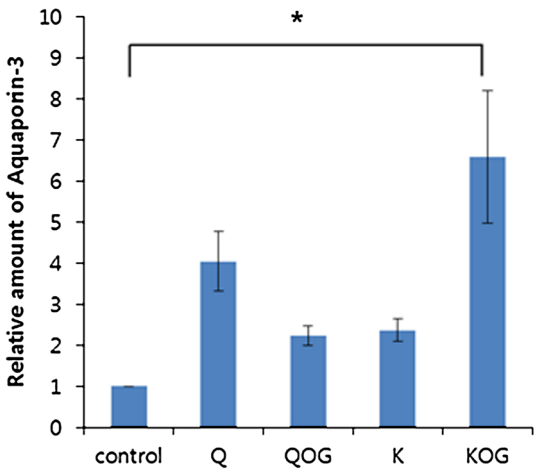


Fig. 4 The effect of flavonols (10 μg/ml) on the mRNA expression level of aquaporin-3 in HaCaT cells. Q, quercetin (**1**); K, kaempferol (**2**); QOG, quercetin-3-*O*-β-D-glucoside (**3**); KOG, kaempferol-3,7-di-*O*-β-D-glucoside (**4**); Cont, DMSO. *p < 0.01

In summary, glucosides of quercetin and kaempferol were prepared in our study, and their biological activities were evaluated for potential cosmeceutical use. Among these, quercetin-3-*O*-β-D-glucoside (**3**) enhanced collagen synthesis by 60 %, while kaempferol-3,7-di-*O*-β-D-glucoside (**4**) showed a promising skin-moisturizing effect with a sixfold induction of AQP3 expression.

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