Original article

Flavonoid glycosides from the leaves of *Uvaria rufa* with advanced glycation end-products inhibitory activity

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Abstract:

Prolonged hyperglycemia in diabetic patients leads to the formation of advanced glycation end-products (AGEs) in their body tissues, which contribute to the development and progression of diabetic complications and aging. Preliminary screening of the ethyl acetate extract of the leaves of *Uvaria rufa* Blume (family Annonaceae) indicated its AGEs inhibitory activity. Fractionation of the extract yielded five flavonol glycosides including rutin **(1)** isoquercitrin **(2)**, kaempferol 3-O-β-D-galactopyranoside **(3)**, astragalin **(4)**, and isoquercitrin-6-acetate **(5)**. Isoquercitrin and its 6-acetate derivative were comparable with the positive control, quercetin, in their ability to inhibit the formation of AGEs in the bovine serum albumin (BSA)-glucose assay, having the 50% inhibitory concentrations (IC_{50}) of 8.4, 6.9 and 10.9 μM, respectively.

Keywords: Advanced glycation end-products inhibition; Annonaceae; Flavonoid glycosides; *Uvaria rufa* Blume

Introduction

Elevated blood glucose level is an important factor in the pathogenesis of several vascular complications in diabetic patients. Prolonged hyperglycemia results in the formation of advanced glycation end-products (AGEs) in body tissues of these patients. The non-enzymatic Maillard reaction between carbonyl group of the reducing sugars and free amino group of proteins leads to the formation of glycated protein called Amadori product. Further rearrangement, oxidation and reduction of the Amadori product yield several AGEs. These complex, irreversible and fluorescent molecules can react with free amino groups nearby, causing protein cross-linking [1, 2]. The cross-linked proteins, such as Collagen and albumin, contribute to the development and progression of pathological conditions found in diabetic patients and in the aging process, including the loss of collagen elasticity and subsequent reduction in arterial and myocardial compliance [3], the binding of modified circulating proteins in the blood to AGE receptors and the activation of inflammatory process, resulting in vascular damage [4].

A powerful AGE inhibitor, aminoguanidine, has been developed as a treatment of diabetic nephropathy. However, reported side effects including vasculitis and abnormalities in liver function were associated with its long-term administration in human and less toxic agents are desirable [5]. Natural compounds, especially flavonoids and polyphenolic substances which are present in common foodstuffs and spices, have been demonstrated to inhibit protein glycation [6-8]. Investigation of their activity on AGEs should therefore offer a potential in the prevention and reduction of diabetic complications.

Uvaria rufa Blume (family Annonaceae) is a climbing shrub found growing in deciduous and evergreen forests throughout Thailand. Its wood and roots are used in traditional Thai medicine to treat fever. Six flavonoids, including 2, 5-dihydroxy-7-methoxy flavanone, 2, 5-dihydroxy-6, 7-dimethoxy flavanone, 5-hydroxy-7-methoxy flavanone, tectochrysin, 6, 7-O, O-dimethylbaicalein and 7-O-methylwogonine, and a number of alkaloids have been isolated from its bark and roots [9-11], whereas several polyoxygenated cyclohexene derivatives were reported as constituents of its aerial parts [12, 13]. In the course of our search for potential AGEs formation inhibitors from Thai annonaceous plants [14], an ethyl acetate (EtOAc) extract of *U. rufa* leaves, at 5 μg/ml, was able to produce 47.9% inhibition against AGEs formation in the preliminary *in vitro* assay. Further fractionation and purification of the extract yielded five flavonol glycosides **1-5.** These compounds were tested for their glycation inhibitory activity, in comparison with the flavonol quercetin **(6)** as positive control.

Material and Methods

Instruments

^{1}H and ^{13}C NMR spectra in DMSO-d_6 were recorded on a Bruker Avance DPX-300 spectrometer at 300 and 75 MHz, respectively, with TMS as internal standard. Preparative MPLC was performed on a Yamazen pump 540 (Yamazen Corporation, Osaka, Japan) with a UV detector (λ 254 nm, Yamazen model PREP. UV-10V) using an Ultra Pack ODS-S-50C (average particle size 50 μm, average pore diameter 120 Å, 37 mm x 300 mm; Yamazen Corporation, Osaka, Japan) column. Analytical HPLC was carried out on a Gilson pump 306 (Gilson Inc., WI, USA) with a UV/VIS-151 detector using a Cosmosil 5C18-AR II (average particle size 5 μm, average pore diameter 120 Å, 4.6 mm x 150 mm; Nacalai Tesque Inc., Kyoto, Japan) column, whereas semi-preparative HPLC was performed using a Cosmosil 5C18-ARII (20 mm x 250 mm; Nacalai Tesque Inc., Kyoto, Japan) column. Fluorescence spectra were recorded on a Hitachi F-2000 fluorescence spectrophotometer (Japan).

Plant material

The leaves of *Uvaria rufa* Blume were collected from Chachoengsao province, Thailand in April 2003, and identified by R. Suttisri. A voucher specimen (RS04031) has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Extraction

The dried, powdered leaves of *Uvaria rufa* (290 g) were repeatedly macerated with hexane (1.5 L x 5),

EtOAc (1.5 L x 5) and MeOH (1.5 L x 5), respectively, to give hexane extract (8 g), EtOAc extract (10 g) and MeOH extract (30 g) after evaporation under reduced pressure at 45°C. The EtOAc extract, which showed the most promising AGEs inhibitory activity, was selected for further fractionation.

Isolation of flavonoids

The EtOAc extract (2.5 g) was subjected to reversed-phase preparative MPLC on an Ultra Pack ODS-S-50C column eluted with a stepwise gradient of MeOH:H_2O (0:1 $\rightarrow$ 4:1; flow rate, 5 ml/min). The eluates were collected and combined according to their analytical HPLC chromatograms on a Cosmosil 5C18-ARII column (mobile phase, acetonitrile:H_2O = 3:17; flow rate, 1 ml/min) into eleven fractions (UR01-UR11). Fraction UR04 (840 mg) was further purified by semi-preparative HPLC using a Cosmosil 5C18-ARII column with a gradient of acetonitrile:H_2O (1:9 $\rightarrow$ 4:1; flow rate, 2 ml/min) as the eluent to give rutin (**1**, 94.3 mg), isoquercitrin (**2**, 184.6 mg), kaempferol 3-O-β-D-galactopyranoside (**3**, 134.9 mg), astragalin (**4**, 107.2 mg) and isoquercitrin-6-acetate (**5**, 37.8 mg).

Rutin [quercetin 3-O-α-L-rhamnopyranosyl (1$\rightarrow$6)-β-D-glucopyranoside; **1***]*

Yellow amorphous powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.53 (1H, *d, J* = 8.4 Hz, H-6′), 7.51 (1H, *br s*, H-2′), 6.83 (1H, *d, J* = 8.4 Hz, H-5′), 6.37 (1H, *br s*, H-8), 6.18 (1H, *br s*, H-6), 5.32 (1H, *d, J* = 7.2 Hz, H-1″), 4.37 (1H, *br s*, H-1‴) and 0.97 (3H, *d, J* = 5.1 Hz, H-6‴); ^{13}C NMR (75 MHz, DMSO-d_6) δ 156.1 (C-2), 133.0 (C-3), 177.0 (C-4), 160.9 (C-5), 98.5 (C-6), 163.8 (C-7), 93.4 (C-8), 156.3 (C-9), 103.7 (C-10), 120.9 (C-1′), 115.0 (C-2′), 144.4 (C-3′), 148.1 (C-4′), 116.0 (C-5′), 121.3 (C-6′), 101.0 (C-1″), 73.9 (C-2″), 76.3 (C-3″), 70.4 (C-4″), 75.8 (C-5″), 66.9 (C-6″), 100.5 (C-1‴), 70.2 (C-2‴), 69.9 (C-3‴), 71.7 (C-4‴), 68.1 (C-5‴), and 17.7 (C-6‴), consistent with reported values [15].

Isoquercitrin (quercetin 3-O-β-D-glucopyranoside; **2***)*

Yellow amorphous powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.58 (1H, *d, J* = 9.0 Hz, H-6′), 7.56 (1H, *br s*, H-2′), 6.83 (1H, *d, J* = 9.0 Hz, H-5′), 6.39 (1H, *d, J* = 1.8 Hz, H-8), 6.18 (1H, *d, J* = 1.8 Hz, H-6) and 5.45 (1H, *d, J* = 6.9 Hz, H-1″); ^{13}C NMR (75 MHz, DMSO-d_6) δ 156.0 (C-2), 133.2 (C-3), 177.2 (C-4), 161.0 (C-5), 98.6 (C-6), 164.0 (C-7), 93.5 (C-8), 156.1 (C-9), 103.9 (C-10), 121.0 (C-1′), 115.1 (C-2′), 144.6 (C-3′), 148.3 (C-4′), 116.1 (C-5′), 121.5 (C-6′), 100.8 (C-1″), 74.1 (C-2″), 76.5 (C-3″), 70.0 (C-4″), 77.5 (C-5″) and 61.0 (C-6″), in agreement with reported data [16].

Kaempferol 3-O-β-D-galactopyranoside **(3)**

Yellow amorphous powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.06 (2H, *d, J* = 8.7 Hz, H-2′, H-6′), 6.85 (2H, *d, J* = 8.7 Hz, H-3′, H-5′), 6.41 (1H, *d, J* = 1.8 Hz, H-8), 6.18 (1H, *d, J* = 1.8 Hz, H-6) and 5.39 (1H, *d, J* = 7.6 Hz, H-1″); ^{13}C NMR (75 MHz, DMSO-d_6) δ 156.5 (C-2), 133.4 (C-3), 177.6 (C-4), 161.3 (C-5), 98.8 (C-6), 164.3 (C-7), 93.8 (C-8), 156.5 (C-9), 104.0 (C-10), 121.0 (C-1′), 131.0 (C-2′), 115.1 (C-3′), 160.0 (C-4′), 115.1 (C-5′), 131.0 (C-6′), 101.8 (C-1″), 71.3 (C-2″), 73.2 (C-3″), 68.0 (C-4″), 75.9 (C-5″) and 60.3 (C-6″), in agreement with reported data [17].

Astragalin (kaempferol 3-O-β-D-glucopyranoside; **4***)*

Yellow amorphous powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.02 (2H, *d, J* = 8.7 Hz, H-2′, H-6′), 6.87 (2H, *d, J* = 8.7 Hz, H-3′, H-5′), 6.43 (1H, *d, J* = 1.8 Hz, H-8), 6.20 (1H, *d, J* = 1.8 Hz, H-6) and 5.44 (1H, *d, J* = 7.2 Hz, H-1″); ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.0 (C-2), 133.1 (C-3), 177.2 (C-4), 156.1 (C-5), 98.7 (C-6), 164.0 (C-7), 93.6 (C-8), 156.2 (C-9), 103.9 (C-10), 120.8 (C-1′), 130.7 (C-2′), 115.0 (C-3′), 159.8 (C-4′), 115.0 (C-5′), 130.7 (C-6′), 100.8 (C-1″), 74.2 (C-2″), 76.4 (C-3″), 69.9 (C-4″), 77.5 (C-5″) and 60.9 (C-6″), consistent with reported values [18].

Isoquercitrin-6-acetate [quercetin 3-(6″-O-acetyl)-β-D-glucopyranoside; **5***]*

Yellow amorphous powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.52 (1H, *d, J* = 7.9 Hz, H-6′), 7.51 (1H, *br s*, H-2′), 6.82 (1H, *d, J* = 7.9 Hz, H-5′), 6.40 (1H, *d, J* = 1.8 Hz, H-8), 6.20 (1H, *d, J* = 1.8 Hz, H-6), 5.36 (1H, *d, J* = 7.1 Hz, H-1″), 4.11 (1H, *dd, J* = 10.5, 2.0 Hz, H-6a″),

3.94 (1H, *dd, J* = 10.5, 6.0 Hz, H-6b″) and 2.49 (3H, *s,* CH_3COO-); ^{13}C NMR (75 MHz, DMSO-d_6) δ 156.4 (C-2), 133.1 (C-3), 177.2 (C-4), 161.1 (C-5), 98.8 (C-6), 164.1 (C-7), 93.6 (C-8), 156.3 (C-9), 103.9 (C-10), 121.1 (C-1′), 115.2 (C-2′), 144.8 (C-3′), 148.5 (C-4′), 116.2 (C-5′), 121.5 (C-6′), 101.0 (C-1″), 74.1 (C-2″), 76.4 (C-3″), 69.6 (C-4″), 74.1 (C-5″), 62.9 (C-6″), 169.8 (-O$\underline{C}$OCH$_3$) and 20.4 (-OCO$\underline{C}$H$_3$), in agreement with literature [19, 20].

Assay for AGEs formation inhibitory activity

The assay for the ability of the fractions and flavonoids to inhibit the glucose-mediated protein glycation and the development of fluorescent AGEs was performed [21]. Each fraction or flavonoid was dissolved in DMSO. The reaction mixtures, containing 400 μg bovine serum albumin (BSA; Waco Pure Chemical Industries, Osaka, Japan), 200 mM glucose, 10 μl of test sample solution or DMSO and 50 mM phosphate buffer (pH 7.4) to a total volume of 500 μl, were incubated at 60 °C for 30 h. A blank, which contained the protein and glucose but no test sample, was kept at 4 °C until measurement. After cooling, aliquots of 250 μl were transferred to 1.5 ml plastic tubes, then, 25 μl of 100% (w/v) trichloroacetic acid (Waco Pure Chemical Industries, Osaka, Japan) were added to each tube and stirred. The supernatant was removed after centrifugation (15,000 rpm) at 4 °C for 4 min and the AGEs-BSA precipitate was dissolved with 1 ml of alkaline phosphate-buffered saline (pH 10). Fluorescence of these solutions was measured on a spectrophotometer at the excitation and emission maxima of 360 and 460 nm, respectively, against the unincubated blank. Percent inhibition of AGEs formation by each sample was calculated as [1-(fluorescence of the sample solution/fluorescence of the control solution)] x 100%. Measurements were performed in triplicate, and the concentration required for a 50% inhibition (IC_{50}) of the fluorescence intensity was determined graphically.

Results and Discussion

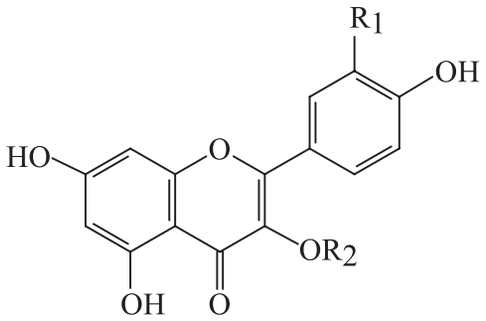
The EtOAc extract of *Uvaria rufa* leaves was separated on a reversed-phase column into 11 fractions. The ability of these fractions to inhibit AGEs formation was evaluated using the BSA-glucose assay, which employed bovine serum albumin as the model protein and glucose as the glycating agent. The results are presented in Table 1. Six fractions (UR03-UR04, UR08-UR11) were able to prevent protein glycation more than 50%.

The most active fraction UR04, which was able to inhibit 61.7% of AGEs formation, yielded five flavonol glycosides i.e. rutin (**1**), isoquercitrin (**2**), kaempferol 3-O-β-D-galactopyranoside (**3**), astragalin (**4**), and isoquercitrin-6-acetate (**5**), upon separation on a semi-preparative HPLC column. These flavonoids were identified by comparison of their ^1H-and ^{13}C-NMR data with literature values (Figure 1). Although a number of flavonoids have been isolated mainly from the root of this plant [10, 11], there had been no previous examination on the contents of its leaves and these flavonol glycosides are reported as constituents of *Uvaria rufa* for the first time. Their AGEs inhibitory activity was evaluated from their ability to reduce the fluorescence formed as an end result of the Maillard reaction. Comparison was made with the flavonoid quercetin, which is known as a potent protein glycation inhibitor [1, 22]. Quercetin is also the aglycone of the isolated glycosides **1, 2** and **5.** These three constituents were evidently more active than the kaempferol glycosides **3** and **4** (Table 2). This is in agreement with previous reports that the 3′-hydroxyl group, present in quercetin but not in kaempferol, contributed to the AGEs inhibitory activity of flavonoids [6] and this activity became stronger as the hydroxyl groups at their 3′-, 4′-, 5′, and 7-positions increased in number [23]. Although there has been a suggestion that glycosylation of the 3-hydroxyl group might increase the activity [23], and, thus, the 3-O-glycoside should be more active than its aglycone, in this study the IC_{50} values for protein glycation inhibition of both glycosides **2** and **5** (8.4 and 6.9 μM, respectively) were comparable to that of quercetin itself (10.9 μM).

Rutin (**1**), which has previously been shown to inhibit AGEs formation [24], displayed lower percent inhibition (41.3%) and, therefore, was less active than quercetin (63.4%) in our model system. The

3-glycosylation of quercetin with a disaccharide (in this case, with rutinose) yielding rutin has been reported to reduce the antioxidant activity relative to its aglycone [25]. AGEs inhibitory activity of natural compounds is considered to be closely associated with their antioxidant capacity [26] and its decrease certainly reflect this relationship.

Both isoquercitrin (**2**) and astragalin (**4**) have previously been isolated from the leaves of *Eucommia ulmoides*, a plant traditionally used in Korea to treat diabetes, and were shown to inhibit protein glycation [27]. However, this is the first report of isoquercitrin-6-acetate (**5**) as possessing the AGEs inhibitory activity.



1: R_1 = OH, R_2 = β-D-Glucose ^{6}-α-L-Rhamnose
2: R_1 = OH, R_2 = β-D-Glucose
3: R_1 = H, R_2 = β-D-Galactose
4: R_1 = H, R_2 = β-D-Glucose
5: R_1 = OH, R_2 = β-D-Glucose-6-acetate
6: R_1 = OH, R_2 = H

Figure 1 Structures of flavonoid glycosides **1-5,** isolated from the leaves of *Uvaria rufa*, and quercetin (**6**)

Table 1 Percent inhibition on AGEs formation of fractions from the EtOAc extract of *Uvaria rufa* leaves (at 2.5 μg/ml)

Fraction	% Inhibition	Fraction	% Inhibition
UR01	18.6	UR07	46.8
UR02	6.0	UR08	53.1
UR03	58.6	UR09	57.8
UR04	61.7	UR10	54.9
UR05	32.8	UR11	53.9
UR06	31.3		

Table 2 Inhibitory activity of flavonoids isolated from the leaves of *Uvaria rufa* on AGEs formation (at 2.5 μg/ml)

Flavonoid	% Inhibition	IC_{50} (μM)
Rutin (**1**)	41.3	-
Isoquercitrin (**2**)	55.2	8.4
Kaempferol 3-O-β-D-galactopyranoside (**3**)	8.5	-
Astragalin (**4**)	16.4	-
Isoquercitrin-6-acetate (**5**)	61.5	6.9
Quercetin (**6**)	63.4	10.9

Conclusion

Five flavonoid glycosides were isolated from the leaves of *Knema furfuracea* for the first time. When subjected to BSA-glucose assay, the flavonoids with hydroxyl groups at both 3′-and 4′-positions in their B-ring were more active than those with 4′-hydroxyl substitution alone. Two of the isolated flavonoids, i.e. isoquercitrin and isoquercitrin-6-acetate, were comparable with quercetin in their ability to inhibit the formation of advanced glycation end-products.

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