Flavonol glycosides isolated from the seeds of *Lens culinaris* inhibit dipeptidyl peptidase IV

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1. Introduction

Dipeptidyl peptidase IV (DPP-IV) is a new target for the treatment of type 2 diabetes mellitus. DPP-IV is the enzyme responsible to the degradation of incretins, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). DPP-IV inhibitors shorten the inactivation of GLP-1 and permit GLP-1 to stimulate insulin release, thereby combating hyperglycemia. [1].

Lens culinaris Medikus is a pulse crop of the family Fabaceae, and its edible seed is known as lentil. Lentil was reported to have diverse biological activities such as antioxidant [2, 3], α-glucosidase–inhibitory [3], anti-inflammatory [11], and anticancer effects [4]. In a previous phytochemical study, phenolics, flavonoids, tannins, saponins, and fatty acids were isolated from *L. culinaris* [2, 5, 6].

As part of our ongoing search for functional resources from mutant cultivars developed by radiation breeding, our research group is cultivating gamma irradiated mutant lines of *L. culinaris* which are developed by various dose of gamma-irradiation from a labeled Cobalt (^{60}Co) source on the seeds. To select the elite cultivar lines through the identification of active components of lentil, the *n*-butanol-soluble fraction of the seeds of *L. culinaris* was investigated and three known flavonol glycosides were isolated. All isolates were experimented with their DPP-IV inhibiting activities, using *in vitro* bioassay.

2. Methods and Results

2.1 General experimental procedures

The 1D NMR experiment was performed on a JNM-ECA 500MHz NMR instrument (JEOL Ltd., Tokyo, Japan). LC/ESIMS was carried out on an Agilent 1200 series system and an Agilent 6120 quadrupole MS system (Agilent Technologies Co., Santa Clara, California, USA). Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F254 (Merck, Darmstadt, Germany), with visualization performed under UV light (254 and 365 nm) and 10% (v/v) sulfuric acid spray followed by heating (200 ℃, 2 min). YMC Gel ODS-A (12 nm, S-150 μm; YMC Co., Kyoto, Japan) and Sephadex LH-20 (Pharmacia Co., Uppsala, Sweden) were used for column chromatography (CC). Kaempferol (≥ 97.0 %, HPLC) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals and solvents used in this study were of analytical grade.

2.2 Extraction and isolation

The dried seeds of *L. culinaris* (8 kg) were pulverized and extracted with 95% EtOH (3 × 15 L) overnight at room temperature. The solvent was evaporated in vacuum to afford a 95% EtOH extract (210 g), whih was then suspended in distilled water (1 L) and partitioned with n-hexane (2 × 3 L), chloroform (2 × 3 L), ethyl acetate (1 L) and n-butanol (1 L), sequentially. The n-butanol-soluble fraction (2.3 g) was subjected to RP-C18 CC (MeOH–water, 1:1 to 1:0, v/v) to yield seven fractions (F01–F07). Fraction F02 (1.5 g) was subjected to RP-C18 CC (MeOH–water, 1:2, v/v) to give three sub-fractions (F0201–F0203). Sub-fraction F0202 (160.8 mg) was chromatographed on a Sephadex LH-20 (100% MeOH), providing **3** (16.5 mg). Sub-fraction F0203 (25.9 mg) was chromatographed on a Sephadex LH-20 (100% MeOH), furnishing **1** (15.0 mg) and **2** (1.0 mg).

*2.3 Isolation of compounds **1**–**3** from the seeds of L. culinaris*

Compounds **1**-**3** were isolated from seeds of L. culinaris. Their structures were identified by spectra analysis data in comparison of their data with published values (Figure 1).

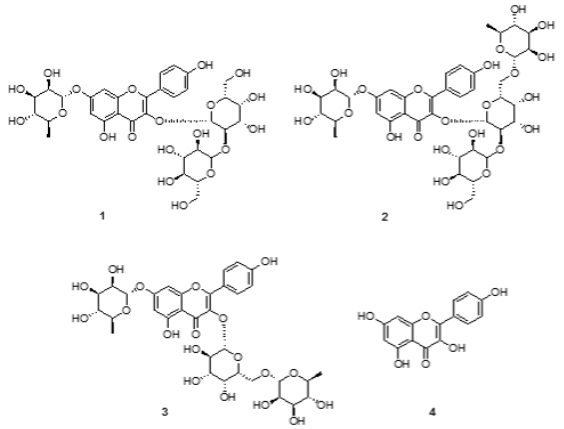

Figure 1. Chemical structures of compounds **1**–**3** isolated from the seeds of *Lens culinaris* and kaempferol (**4**).

Compound **1** (kaempferol-3-*O*-*β*-gulcopyranosyl-(1→2)-*β*-galactopyranosyl-7-*O*-*α*-rhamnopyranoside). Pale yellow solid. ESI-MS m/z 756.6 $[M]^+$. ^{1}H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 8.11 (2H, d, J = 9.0, H-2' and H-6'), 6.90 (2H, d, J = 9.0, H-3' and H-5'), 6.76 (1H, br s, H-8), 6.46 (1H, br s, H-6), 5.56 (1H, br s, H-1 of 7-*O*-Rha), 5.41 (1H, d, J = 7.5, H-1" of 3-*O*-Gal), 4.75 (1H, d, J = 7.5 Hz, H-1 of 2"-*O*-Glc), 1.24 (3H, d, J = 5.0, H-6 of 7-*O*-Rha) [7]. ^{13}C NMR data: see Table 1.

Compound **2** (kaempferol-3-*O*- *β*-gulcopyranosyl-(1→2)-[α-rhamnopyranosyl(1→6)]-*β*-galactopyranosyl-7-*O*-*α*-rhamnopyranoside). Pale yellow solid. ESI-MS m/z 902.8 $[M]^+$. ^{1}H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 8.06 (2H, d, J = 9.1, H-2' and H-6'), 6.91 (2H, d, J = 9.1, H-3' and H-5'), 6.75 (1H, br s, H-8), 6.48 (1H, br s, H-6), 5.56 (1H, br s, H-1 of 7-*O*-Rha), 5.36 (1H, d, J = 7.5, H-1" of 3-*O*-Gal), 4.73 (1H, d, J = 7.5 Hz, H-1 of 2"-*O*-Glc), 4.47 (1H, br s, H-1 of 6"-O-Rha), 1.25 (3H, d, J = 5.5, H-6 of 7-*O*-Rha), 1.06 (3H, d, J = 5.5, H-6 of 6"-O-Rha) [8]. ^{13}C NMR data: see Table 1.

Compound **3** (kaempferol-3-*O*-*α*-rhamnosyl(1→6)-*O*-*β*-galactopyranoside-7-*O*-*α*-rhamnopyranoside). Pale yellow solid. ESI-MS m/z 740.6 $[M]^+$. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 1H NMR (500 MHz, CD3OD, δ , ppm, J/Hz): 8.12 (2H, d, J = 8.5, H-2' and H-6'), 6.87 (2H, d, J = 8.5, H-3' and H-5'), 6.77 (1H, d, J = 1.5, H-8), 6.48 (1H, d, J = 1.5, H-6), 5.57 (1H, br s, H-1 of 7-*O*-Rha), 5.09 (1H, d, J = 7.5, H-1" of 3-*O*-Gal), 4.51 (1H, br s, H-1 of 6"-*O*-Rha), 1.26 (3H, d, J = 6.0, H-6 of 7-*O*-Rha), 1.18 (3H, d, J = 6.0, H-6 of 6"-*O*-Rha) [8]. ^{13}C NMR data: see Table 1.

*2.4 DPP-IV inhibitory activity of compounds **1**-**4***

DPP-IV activity was analyzed using a DPP-IV inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI, USA) which provides a fluorescence-based method for screening DPP-IV inhibitors. The assay uses the fluorogenic substrate, Gly-Pro-Aminomethyl-coumarine (AMC), to measure DPP-IV activity. Cleavage of the peptide bond by DPP releases the free AMC group, resulting in fluorescence that can be analyzed using an excitation wavelength of 350–360 nm and an emission wavelength of 450–465 nm. The percent inhibition was expressed as ([DPP-IV level of vehicle-treated control − DPP-IV level of test samples]/DPP-IV level of vehicle-treated control) × 100. DDP-IV was tested for the absence or presence of compounds **1**-**3** (5, 10, 25, and 50 μM) and the commercial compound with an aglycone structure of **1**-**3**, kaempferol (**4**) (6.25, 12.5, 25, 50, and 100 μM). As results, compounds **1**-**4** inhibited the DPP-IV activity in a concentration-dependent manner (Fig. 2). Their IC_{50} value, the sample concentration resulting in 50% inhibition of DPP-IV activity, was determined by linear standard curve and calculated based on the molecular mass of each compound. Compounds **1**-**4** showed inhibitory activity with IC_{50} values of 27.89 ± 1.29, 36.52 ± 0.78, 37.01 ± 1.40, and 51.9 ± 4.83 μM, respectively. Sitagliptin was used as a positive control and exhibited its inhibitory activity with IC_{50} values of 70.76 ± 4.83 nM. Therefore, our results suggest the potential of **1**–**3** as naturally occurring agents for treating DPP-IV–mediated hyperglycemia and type 2 diabetes mellitus, although further studies are required to clarify their mechanisms of action using *in vitro* and *in vivo* models.

Table 1. ^{13}C NMR data of **1**-**3** (125 MHz, δ, ppm) [7,8].

C atom	**1** (CD_3OD)	**2** (CD_3OD)	**3** (DMSO-d_6)
2	158.2	158.4	157.0
3	133.7	133.7	133.0
4	178.4	178.4	177.6
5	161.6	161.6	160.8
6	99.3	99.3	99.3
7	162.3	162.3	161.6
8	94.5	94.5	94.8
9	156.8	156.8	156.0
10	106.1	106.1	105.5
1'	121.3	121.3	120.6
2'	131.2	131.2	131.1
3'	115.0	115.0	115.1
4'	160.4	160.4	160.8
5'	115.0	115.0	115.1
6'	131.2	131.2	131.1
3-*O*-Gal			
1"	99.9	99.6	101.8
2"	81.2	81.2	71.1
3"	74.3	74.3	72.9
4"	69.9	69.9	68.2
5"	77.5	75.8	73.5
6"	61.5	66.8	65.0
2"-*O*-Gal			
1	103.4	103.4	
2	75.8	75.8	
3	76.6	76.6	
4	70.0	70.0	
5	76.9	76.9	
6	61.3	61.3	
6"-*O*-Rha			
1		100.8	100.0
2		70.8	70.4
3		71.0	70.6
4		72.5	71.9
5		68.4	67.9
6		16.5	17.9
7-*O*-Rha			
1	98.6	98.6	98.4
2	70.4	70.4	70.1
3	70.8	70.8	70.2
4	72.3	72.3	71.6
5	70.1	70.1	69.8
6	16.8	16.8	17.9

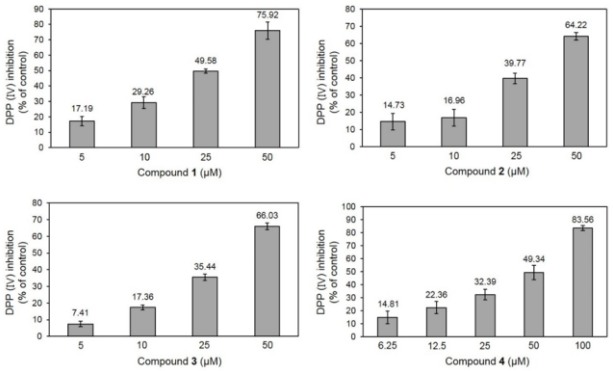

Figure 2. Effects of compounds **1**–4 on dipeptidyl peptidase IV (DPP-IV) activity. Values are presented as the mean ± SD of three independent experiments.

3. Conclusions

In conclusion, we reported the inhibitory effects of flavonol glycosides, particularly compounds **1**–**3** isolated from the seeds of *L. culinaris*, against human recombinant DPP-IV for the first time. The present result can be applied to the quality assurance of gamma irradiated mutant cultivar lines of *L. culinaris*, and also suggest that the selected elite mutant cultivars of *L. culinaris* serves as a promising source for natural anti-diabete compounds for application in the field of functional foods.

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