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Author(s)	Noda, Kota; Kato, Eisuke; Kawabata, Jun
Citation	Natural Product Communications, 12(8), 1259-1261 https://doi.org/10.1177/1934578X1701200828
Issue Date	2017-08-01
Doc URL	http://hdl.handle.net/2115/75147
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Type	article (author version)
File Information	NPC2017_Achillea_millefolium.pdf



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Intestinal α -Glucosidase Inhibitors in *Achillea millefolium*

Kota Noda, Eisuke Kato,* and Jun Kawabata

Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan.

eikato@chem.agr.hokudai.ac.jp

Received: January XX, 2016; Accepted: XX, 2016

Achillea millefolium is a plant used as a component of anti-diabetic preparation but the bioactive compounds responsible for its use are not known. Inhibition of intestinal α -glucosidase is a preferable effect for prevention and treatment of diabetes, and *A. millefolium* extract showed inhibitory activity against the enzyme. Activity-guided separation of the extract gave four mono- or di-caffeoylquinic acids as the isolate. Quantitation of these four caffeoylquinic acids and the activity of the isolates suggested that these are the major contributor for the α -glucosidase inhibitory activity in the extract. However also, the presence of unidentified, minor, but potent α -glucosidase inhibitor in the isolate was also suggested.

Keywords: diabetes, intestinal α -glucosidase, maltase, glucosidase inhibitor, caffeoylquinic acid

Diabetes mellitus (DM) is a worldwide problem due to increase of the patient number year to year. DM is a chronic disease exhibiting high level of blood sugar due to either decreases in insulin production or sensitivity to insulin.

Intestinal α -glucosidase is an essential enzyme for the decomposition and absorption of carbohydrates consumed as food. However, its inhibition can attenuate the rapid elevation of blood sugar level after meal consumption and therefore it is utilized for the treatment and prevention of DM.

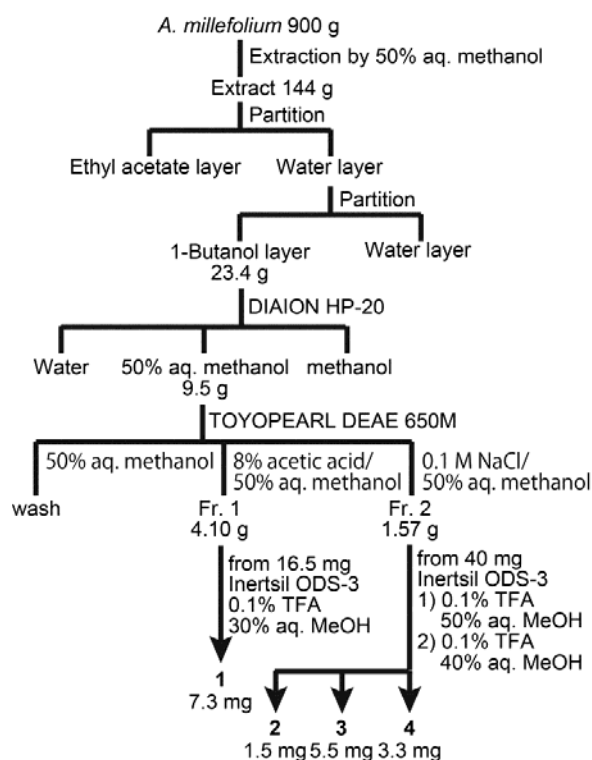
Achillea millefolium, commonly named yarrow, is a perennial herbal plant belonging to Asteraceae family. The plant is native to the regions of Northern Hemisphere including Eastern Asia to Europe. The plant is commonly consumed as an herbal tea or prepared as an essential oil. The traditional use of this plant includes the treatment of inflammatory and spasmodic gastro-intestinal disorders, for wound healing, skin inflammations or as an appetite-enhancing drug,^{1,2} and also as a component of antidiabetic preparation.^{3,4}

Recent scientific studies on this plant revealed sesquiterpenes as an active ingredient related to the antiphlogistic activity,⁵ dicaffeoylquinic acids as the choleric substances,⁶ which are assumed to be related to anti-inflammatory properties and problems in digestive tracts. However, the active compound related to the antidiabetic property of *Achillea millefolium* has not been reported.

Here, we searched for the intestinal α -glucosidase inhibitor from the extract of *Achillea millefolium*, and found several dicaffeoylquinic acids as the active component, which may contribute at least in part to the antidiabetic property of this plant.

The dried aerial part of *Achillea millefolium* was extracted by aqueous methanol. The extract showed 55% inhibition at 1.6 mg/mL against rat intestinal α -glucosidase and was separated with a guidance of the activity. The extract was partitioned between water, ethyl acetate and 1-butanol. The 1-butanol soluble part was further purified by DIAION HP-20 (Mitsubishi Chemical Co., Tokyo), and TOYOPEARL DEAE 650M (Tosoh Co., Tokyo) column chromatography. The obtained fractions were finally separated by

HPLC using Inertsil ODS-3 (GL Science Co., Tokyo) column to obtain compound 1-4 (Scheme 1).



Scheme 1. Isolation procedure of intestinal α -glucosidase inhibitors from *Achillea millefolium*

Each compound was analyzed by ¹H-NMR and mass spectrometry to determine the structure. ¹H-NMR spectrum revealed that these are caffeoylquinic acids and MS analysis indicated that 1 is a mono-caffeoylquinic acid and 2-4 are di-caffeoylquinic acids. Comparison of the ¹H-NMR spectrum with the possible structures revealed the structure to be chlorogenic acid (5-*O*-caffeoylquinic acid, 1), 3,4-di-

O-caffeoylquinic acid (**2**), 3,5-di-*O*-caffeoylquinic acid (**3**), and 1,5-di-*O*-caffeoylquinic acid (**4**) (Figure 1).^{7,8}

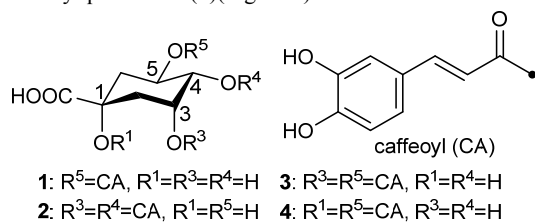


Figure 1. Structures of the compound in peak 1-4

An inhibitory activity of **1-4** was next measured (Figure 2). Among the four caffeoylquinic acids, **2** showed the highest activity with 69% inhibition at 0.5 mM. Compounds **3** and **4** showed weaker activity compared to **2** and chlorogenic acid (**1**) showed the least activity.

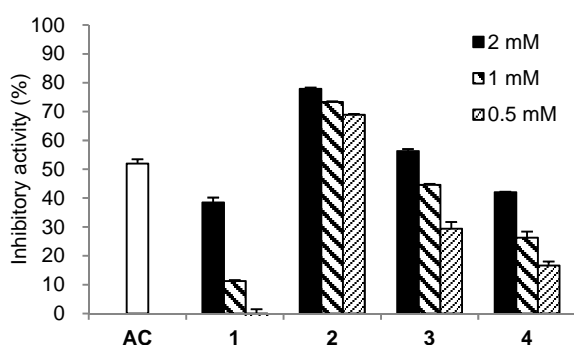


Figure 2. α -Glucosidase inhibitory activity of the isolated compounds. Acarbose (AC, 0.5 μ M) was used as a positive control. Error bars indicate the SEM.

To evaluate a contribution of **1-4** in the activity of the *A. millefolium* extract, a concentration of each compound in the extract was measured by HPLC (Table 1). Then, an inhibitory activity of the individual compounds, mixture of the compounds, and the extract was compared at the concentration adjusted according to Table 1 (Figure 3). The mixture shows slightly weaker activity compared to an inhibitory activity of the extract, indicating that these isolated compounds are the major contributor to the inhibition of α -glucosidase by the *A. millefolium* extract. However, when the activity was compared with the previous reports, there is another speculation.^{8,10}

Table 1: Content of the isolated compounds in *Achillea millefolium* and the extract.

Compounds	mg/g in	mM in
	<i>A. millefolium</i>	1.6 mg/mL extract
1	2.18	0.060
2	0.83	0.016
3	4.85	0.094
4	0.98	0.019

α -Glucosidase inhibitory activity of di-caffeoylquinic acids are shown in several articles.⁸⁻¹⁰ Reported IC₅₀ values are 1-2 mM, depending on the assay procedure, which is in reasonable comparison with the current measured inhibitory activity for compound **3** and **4**. In comparison, compound **2** in the current study shows quite high activity compared to the reported IC₅₀ values (35% at 0.016 mM, Figure 3). Therefore, the fact suggests there may be a contribution of minor component included in **2** for the activity.

The α -glucosidase inhibitory activity of caffeoylquinic acids are known to highly depend on the number of caffeoyl groups attached

to the quinic acid moiety. Tri-caffeoylquinic acids are reported to show much lower IC₅₀ value compared to the di-caffeoylquinic acids.^{9,10} Since di- and tri-caffeoylquinic acids are related natural products, we analyzed the tested sample by MS to check if tri-caffeoylquinic acid is included. However no peaks were identified indicating the presence of tri-caffeoylquinic acid.

In conclusion, α -glucosidase inhibitor in *A. millefolium* extract was investigated in relationship with the plant use as a component of anti-diabetic preparation. *A. millefolium* contained several caffeoylquinic acids (**1-4**) which are reported as α -glucosidase inhibitory compound. The mixture of these compounds showed comparable activity to the extract. However, the activity of **2** was much stronger than that in the previous reports. Therefore, we presume that there may be a more potent inhibitor in the extract of *A. millefolium*.

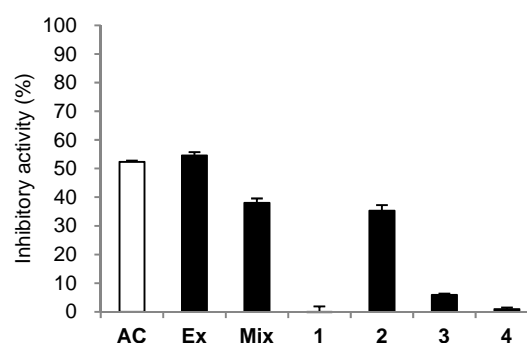


Figure 3. α -Glucosidase inhibitory activity of the extract (1.6 mg/mL) and the individual/ mixture of the isolated compounds. Acarbose (AC, 0.5 μ M) was used as a positive control. Mixture consists of **1** (0.060 mM), **2** (0.016 mM), **3** (0.094 mM), and **4** (0.019 mM). Individual compounds are tested at the same concentration in the mixture. Error bars indicate the SEM.

Experimental

General: All commercially available chemicals were purchased from Wako Pure Chem. Ind. Ltd., unless otherwise noted. *Achillea millefolium* was purchased from Hyakka-Saen.Co.Ltd. Structures of the compounds were determined by NMR and Mass spectrometry. Bruker AMX500 or Jeol JNM-EX 270 was used to obtain NMR spectrum and residual solvent peak was used as an internal standard (methanol-*d*₄ 3.31 ppm). LCT-premier (Waters Co.) was used to obtain mass spectrum. Absorbance was measured by SynergyTM MX (Bio-tech Instruments Inc.) microplate reader.

Isolation: Powdered *Achillea millefolium* (900 g) was extracted with 50% aq. methanol for 24 hr. The extract (144 g) was dispersed in water and partitioned between ethyl acetate and then between 1-butanol. The 1-butanol layer (23.4 g) was concentrated and the residual aq. solution was separated by DIAION HP-20 column chromatography with stepwise elution (water, 50% aq. methanol, and methanol). The 50% aq. methanol eluate (9.5 g) was absorbed to TOYOPEARL DEAE 650 and washed by 50% aq. methanol. The gel was eluted by 8% acetic acid/50% aq. methanol (Fr. 1, 4.10 g) and then by 0.1 M sodium chloride/50% aq. methanol (Fr. 2, 1.57 g). Fr. 1 (16.5 mg) was purified by HPLC (Inertsil ODS-3, 30% aq. methanol with 0.1% trifluoroacetic acid (TFA)). A peak eluted at tR = 31.7 min was collected to obtain compound **1** (7.3 mg). Fr. 2 (40 mg) was purified by HPLC (Inertsil ODS-3, 50% aq. MeOH with 0.1% TFA) and a peak eluted at tR = 18.0 min was collected. This peak (18.6 mg) was further purified by HPLC (Inertsil ODS-3, 40% aq. MeOH with 0.1% TFA) and the peaks eluted at tR = 74.4, 82.9, 102.5 min was collected to obtain compound **2** (1.5 mg), **3** (5.5 mg), and **4** (3.3 mg).

Compound data

chlorogenic acid (**1**): yellow powder; ESI-MS (negative): m/z 353 [M-H]⁻; ¹H-NMR (methanol-*d*₄, 500 MHz): 2.00-2.10 (2H, m), 2.14-2.25 (2H, m), 3.73 (1H, dd, $J = 3.2, 8.5$ Hz), 4.17 (1H, br s), 5.30-5.37 (1H, m), 6.27 (1H, d, $J = 16.0$ Hz), 6.78 (1H, d, $J = 6.1$ Hz), 6.95 (1H, dd, $J = 2.1, 6.1$ Hz), 7.05 (1H, d, $J = 2.1$ Hz), 7.56 (1H, d, $J = 16.0$ Hz) ppm. The data was compared with the commercial chlorogenic acid.

3,4-di-*O*-caffeoylquinic acid (**2**)⁸: yellow powder; ESI-MS (negative): m/z 515 [M-H]⁻; ¹H-NMR (methanol-*d*₄, 500 MHz): 2.06-2.40 (4H, m), 4.30-4.40 (1H, m), 5.01 (1H, dd, $J = 3.1, 8.4$ Hz), 5.62-5.66 (1H, m), 6.25 (1H, d, $J = 15.8$ Hz), 6.29 (1H, d, $J = 15.8$ Hz), 6.73 (1H, d, $J = 8.2$ Hz), 6.77 (1H, d, $J = 8.2$ Hz), 6.88 (1H, br d, $J = 8.2$ Hz), 6.93 (1H, br d, $J = 8.2$ Hz), 7.03 (1H, br s), 7.04 (1H, br s), 7.55 (1H, d, $J = 15.8$ Hz), 7.58 (1H, d, $J = 15.8$ Hz) ppm.

3,5-di-*O*-caffeoylquinic acid (**3**)⁸: white powder; ESI-MS (negative): m/z 515 [M-H]⁻; ¹H-NMR (methanol-*d*₄, 500 MHz): 2.15-2.35 (4H, m), 3.98 (1H, dd, $J = 2.8, 7.3$), 5.37-5.41 (1H, m), 5.42-5.45 (1H, m), 6.35 (1H, d, $J = 16.0$ Hz), 6.27 (1H, d, $J = 16.0$ Hz), 6.78 (2H, d, $J = 8.2$ Hz), 6.96 (1H, br d, $J = 8.2$ Hz), 6.98 (1H, dd, $J = 1.6, 8.2$ Hz), 7.07 (2H, br s), 7.58 (1H, d, $J = 16.0$ Hz), 7.62 (1H, d, $J = 16.0$ Hz) ppm.

1,5-di-*O*-caffeoylquinic acid (**4**)⁷: yellow powder; ESI-MS (negative): m/z 515 [M-H]⁻; ¹H-NMR (methanol-*d*₄, 500 MHz): 2.06 (1H, dd, $J = 9.1, 12.4$ Hz), 2.42 (1H, br d, $J = 12.4$ Hz), 2.49 (1H, br d, $J = 12.4$ Hz), 2.57 (1H, br d, $J = 12.4$ Hz), 3.77 (1H, dd, $J = 3.1, 7.9$ Hz), 4.27-4.31 (1H, m), 5.36-5.42 (1H, m), 6.27 (1H, d, $J = 15.8$ Hz), 6.31 (1H, d, $J = 15.5$ Hz), 6.78 (2H, d, $J = 8.2$ Hz), 6.96 (2H, d, $J = 8.2$ Hz), 7.06 (2H, s), 7.59 (1H, d, $J = 15.8$ Hz), 7.59 (1H, d, $J = 15.5$ Hz) ppm.

α -Glucosidase inhibitory activity: Rat intestinal acetone powder (1 g, Sigma-Aldrich Co.) was homogenized in 20 mL of 0.1 M phosphate buffer (pH 7.0, 5 mM EDTA), centrifuged (15,000 \times 10 min) and the supernatant was used as the crude enzyme. The reaction mixture consisting from the crude enzyme (20 μ L), substrate (8 mM maltose in phosphate buffer, 25 μ L) and the test sample (dissolved in 50% aq. DMSO, 5 μ L) was mixed and incubated at 37 °C for 20 min. The reaction was stopped by adding 75 μ L of 2 M Tris-HCl buffer (pH 7.0), diluted with the phosphate buffer (375 μ L) and then passed through a short column of Cosmosil 75C18-OPN (Nacalai Tesque, Inc.) to remove the test sample. The amount of liberated glucose was measured by Glucose CII-test Wako and compared with the control value to determine the inhibitory activity. Each experiment was done in duplicate and repeated at least twice. The average of experiments is shown in the figure.

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