SUPPLEMENTARY MATERIAL

Glutaminase Inhibitory Activity of Umbelliferone Isolated from Kabosu (Citrus sphaerocarpa Hort. ex Tanaka)

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Abstract
Kabosu (Citrus sphaerocarpa Hort. ex Tanaka) fruits have pleasant and fresh odors and have been used as raw materials for vinegar, seasonings, jams, marmalades and juices in Japan. The n-butanol extracts from kabosu fruits were prepared and a component in the extract was purified by column chromatography and HPLC to afford compounds 1-3. Three compounds, 5-(hydroxymethyl)-2-furaldehyde (1), umbelliferone (2) and oxypeucedanin hydrate (3), have been isolated from kabosu, and the structures of compounds 1-3 were elucidated by 1D and 2D NMR as well as EI-MS. Compound 2 exhibited potent glutaminase inhibitory activity with an IC₅₀ value of 1.33 mM. This is the first report on glutaminase inhibitory activity of 2 and the isolation of three compounds 1-3 from kabosu fruits.

Keywords: Citrus sphaerocarpa, umbelliferone, 5-(hydroxymethyl)-2-furaldehyde, oxypeucedanin hydrate, glutaminase inhibitor
Experimental

General experimental procedures
The $^1$H NMR and $^{13}$C NMR spectra were recorded using a Bruker AVANCE™ III 600 (Karlsruhe, Germany). Coupling constants are expressed in hertz, and chemical shifts are given on a $\delta$ (ppm) scale with tetramethylsilane (TMS) as an internal standard. EI-MS spectra were recorded using a Shimadzu GCMS-QP2010 Plus mass spectrometer (Kyoto, Japan). Column chromatography was performed on 75 - 150 μm MCI-gel CHP20P (Mitsubishi Chemical Co. Ltd., Tokyo, Japan) and 40 - 63 μm silica gel 60 (Merck KGaA, Darmstadt, Germany).

Chemicals
Dimethylsulfoxide (DMSO), DON, L-glutamine, Nessler’s reagent and quercetin were purchased from FUJIFILM Wako Pure Chemical Corporation, Ltd (Osaka, Japan). Glutaminase (Grade V from Escherichia coli, 50-200 units/mg protein composition) was purchased from Sigma-Aldrich Co. (St. Louis, USA). DON was used as a positive control, since it had already been reported to show glutaminase inhibitory and anticancer activities (Thangavelu et al. 2014) and neuroprotective effects (Takeuchi et al. 2008). Quercetin was used as a control, since it was present in most of the plants including fruits and vegetables having antioxidant and anticancer activities and neuroprotective effects (Elizabeta et al. 2019) but was not known about glutaminase inhibitory activity.

Materials
Mature kabosu fruits (2.0 kg) were collected at Oita Prefecture in December 2017. The collected samples were identified by Prof. Hitoshi Yoshimitsu. A voucher specimen (NGAS201712) was deposited at the herbarium of Nakamura Gakuen University. The fruit sample was washed and divided into four equal parts. The sample was homogenized using an Iwatani mixer grinder (Osaka, Japan) at the medium-speed setting for 30 s and the resulting homogenate was filtered through four layers of cheesecloth.

Extraction and isolation procedures
The filtrate (660 mL) from mature kabosu fruits was extracted three times with $n$-butanol (1 L). $n$-Butanol was chosen as the extracting solvent, since $n$-butanol residue (0.47 g/100 mL filtrate) was higher yield than MeOH residue (0.44 g) and the $n$-butanol residue also showed higher glutaminase inhibitory activity by 45% than the MeOH residue by 38% at a concentration of 10 mg/mL. The combined
solvents were concentrated in vacuo. The resulting n-butanol residue (3.13 g) was first fractionated using a column (50 X 300 mm) of MCI gel CHP 20P eluted with the solvent system distilled water–MeOH, three fractions were obtained. Fraction 2 (0.19 g) eluted with 60% MeOH was fractionated by column chromatography on silica gel 60 (30 X 285 mm, CHCl3–MeOH). Fraction 2-2 (26.5 mg) eluted with 60% MeOH was purified by semipreparative HPLC using a column (10 X 250 mm, Cosmosil cholesterol, Nacalai Tesque, Inc., Kyoto, Japan) eluted with 55% MeOH at a flow rate of 0.8 mL/min to obtain compound 1 [1.2 mg: 0.00006% fresh weight (FW)]. Fraction 3 (0.43 g) eluted with MeOH was fractionated by column chromatography on silica gel 60 (30 X 285 mm, CHCl3–MeOH). Fraction 3-2 (72 mg) eluted with 10% MeOH was purified by semipreparative HPLC using a column (10 X 250 mm, Cosmosil cholesterol) eluted with 55% MeOH at a flow rate of 0.8 mL/min to obtain compounds 2 (3.7 mg: 0.00019% FW) and 3 (1.1 mg: 0.00006% FW).

Spectral data of isolated compounds

Compound 1 [5-(hydroxymethyl)-2-furaldehyde]: Compound 1 was yellowish brown powder. 1H NMR (600 MHz, pyridine-d5) δ: 4.51 (2H, s, H-7), 5.60 (1H, br.s, OH), 6.61 (1H, d, J = 3.5 Hz, H-3), 7.50 (1H, d, J = 3.5 Hz, H-4), 9.54 (1H, s, H-6). 13C NMR (150 MHz, pyridine-d5) δ: 55.88 (C-7), 109.68 (C-4), 124.45 (C-3), 151.69 (C-2), 162.11 (C-5), 177.98 (C-6). MS (EI): m/z (%) = 126 (68) [M+] , 109 (44), 97 (85), 81 (20), 69 (41), 53 (32), 44 (100), 41 (86).

Compound 2 (Umbelliferone): Compound 2 was pale brown powder. 1H NMR (600 MHz, pyridine-d5) δ: 6.23 (1H, d, J = 9.0 Hz, H-3), 7.03 (1H, d, J = 1.1 Hz, H-8), 7.04 (1H, dd, J = 8.7, 1.1 Hz, H-6), 7.40 (1H, d, J = 8.7 Hz, H-5), 7.67 (1H, d, J = 9.0 Hz, H-4). 13C NMR (150 MHz, pyridine-d5) δ: 103.06 (C-8), 111.86 (C-4a), 111.93 (C-3), 113.73 (C-6), 129.83 (C-5), 144.23 (C-4), 156.54 (C-8a), 161.23 (C-2), 162.60 (C-7). MS (EI): m/z (%) = 162 (90) [M+] , 134 (100), 105 (29), 78 (35), 63 (12), 51 (26), 43 (8).

Compound 3 (Oxypeucedanin hydrate): Compound 3 was white powder. 1H NMR (600 MHz, pyridine-d5) δ: 1.23 (3H, s, H-14), 1.29 (3H, s, H-15), 3.81 (1H, dd, J = 3.1, 9.3 Hz, H-12), 4.38 (1H, dd, J = 9.3, 10.9 Hz, H-11), 4.79 (1H, dd, J = 3.1, 10.9 Hz, H-11), 6.28 (1H, d, J = 9.7 Hz, H-3), 7.21 (1H, s, H-8), 7.22 (1H, d, J = 2.4 Hz, H-9), 7.79 (1H,
d, $J = 2.4$ Hz, H-10), 8.43 (1H, d, $J = 9.7$ Hz, H-4). $^{13}$C NMR (150 MHz, pyridine-$d_5$) $\delta$: 24.84 (C-14), 27.24 (C-15), 72.70 (C-13), 75.89 (C-11), 78.17 (C-12), 94.72 (C-9), 106.31 (C-8), 108.43 (C-4a), 113.02 (C-3), 115.45 (C-6), 141.75 (C-4), 146.85 (C-10), 150.85 (C-5), 153.96 (C-8a), 159.91 (C-7), 163.33 (C-2). MS (EI): m/z (%) = 304 (27) [M$^+$], 203 (100), 174 (26), 145 (12), 118 (6), 89 (10), 59 (56), 43 (27).

Glutaminase inhibitory assay

Glutaminase inhibitory activity was assayed according to the modified method of Elshafei et al. (2014). The assay mixture (0.5 mL) contained 0.1 mL of a sample solution in 5% DMSO, 0.1 mL of 0.1 M acetate buffer (pH 4.9), 0.1 mL of enzyme solution (2.0 U/mL glutaminase in the same buffer) and 0.2 mL of substrate solution (0.04 mM L-glutamine in the same buffer), mixed and incubated at 37°C for 30 min. The reaction was stopped by adding 0.5 mL of 0.5M H$_2$SO$_4$ solution. The precipitated protein was removed by centrifugation (3000 rpm, 10 min) and 0.2 mL of supernatant was added to 3.8 mL of distilled water. Thereafter, 0.5 mL of Nessler’s reagent was added, and the absorbance was measured at 420 nm within 10 min. The percentage inhibition of the glutaminase inhibitory activity was calculated according to the following equation: the inhibitory activity (%) = $[\frac{(\text{Control Abs} - \text{Control blank Abs}) - (\text{Sample Abs} - \text{Sample blank Abs})}{\text{Control Abs} - \text{Control blank Abs}}] \times 100$, where control is the activity of the enzyme with distilled water instead of sample solution and blank is the activity without the enzyme. DON and quercetin were used as controls (Thangavelu et al. 2014; Elizabeta et al. 2019).

Data analysis

All experiments were performed in triplicate and the data were expressed as means. The 50% inhibitory concentration (IC$_{50}$) values (mM) obtained from log dose inhibition curves are expressed as the mean. Duncan’s multiple-range test was used to assess the statistical significance ($p<0.05$).
Table S1. Inhibitory effect of 1-3, DON and quercetin against glutaminase.

| Compound | IC$_{50}$ (mM) |
|----------|----------------|
| 1        | n.a.           |
| 2        | 1.33$^b$       |
| 3        | n.a.           |
| DON      | 0.57           |
| Quercetin| n.a.           |

Statistical differences were shown as alphabetic letters.
The different letters represented statistical differences, p < 0.05.
n.a.: Not available because of low activity.