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Inhibitors of Xanthine Oxidase from the Flowers and Buds of *Daphne genkwa*[1)]

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Xanthine oxidase (XO) inhibitors were isolated from the flowers and buds of *Daphne genkwa* SIEB. *et* ZUCC. (Thymelaeaceae) and were identified as genkwanin (**1**), apigenin (**2**), luteolin 7-methyl ether (**3**) and luteolin (**4**). Compounds **2** and **4** showed particularly strong inhibitory activity against XO. The modes of inhibition by **2** and **4** with respect to xanthine as a substrate were of mixed type. This is the first report of isolation of **3** and **4** from these flowers and buds.

Keywords——xanthine oxidase; inhibitor; mixed type inhibition; xanthine; *Daphne genkwa*; thymelaeaceae; flavonoid; apigenin; luteolin

The purpose of this study was not only to find and develop new medicinal drugs but also to examine the effects of traditional oriental drugs. Xanthine oxidase (XO, EC 1.2.3.2) oxidizes hypoxanthine to xanthine and finally to uric acid, and the accumulated uric acid causes hyperuricacidemia associated with gout. Thus, specific inhibitors of XO are expected to be therapeutically useful for the treatment of gout.[2)]

In screening test *in vitro* aiming to find XO inhibitors among many crude oriental drugs and plant materials, the flowers and buds of *Daphne genkwa* SIEB. *et* ZUCC. (Japanese name "genka," Thymelaeaceae) were found to possess a strong inhibitory effect on this enzyme. The flowers and buds of *Daphne genkwa* have been used as purgatives, hydragogues and expectorants. The constituents of the flowers and buds hitherto reported include genkwanin (**1**), apigenin (**2**), sitosterol, benzoic acid and genkwadaphnin, which is an antileukemic principle.[3)] In this paper, we describe the isolation of XO inhibitors from the flowers and buds of *Daphne genkwa*.

Results and Discussion

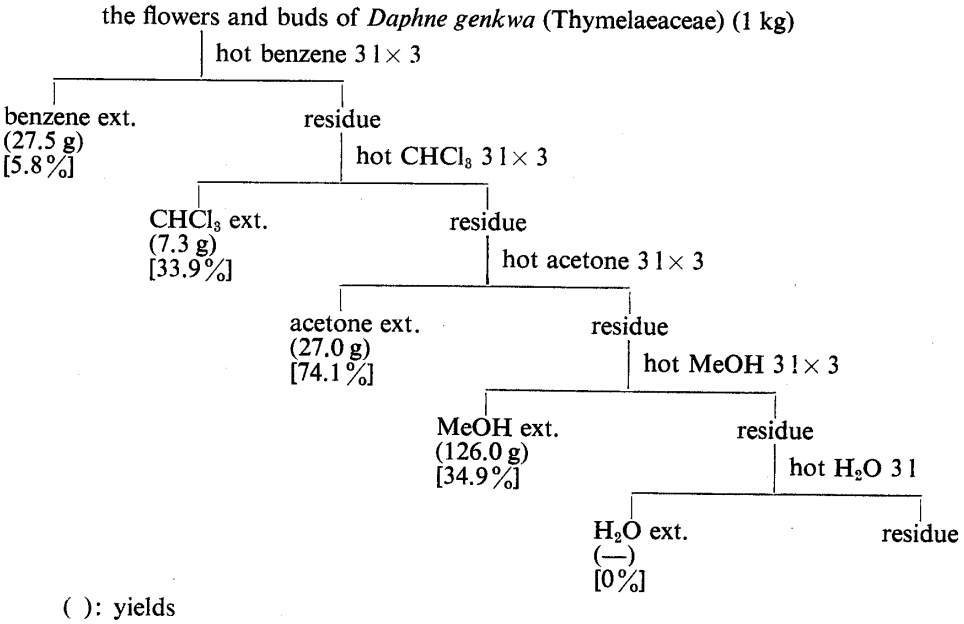
The activity of XO towards xanthine as a substrate was assayed spectrophotometrically at 290 nm by the method of Kalckar[4)] with a modification. The flowers and buds of *Daphne genkwa* were extracted with benzene, chloroform, acetone, methanol and water under reflux as shown in Chart 1. The inhibitory activities of each extract against XO and the yields of each extract are also shown in Chart 1. The most active acetone extract was extracted again with chloroform, ethyl acetate, acetone and methanol under reflux as shown in Chart 2. The inhibitory activities against XO and the yields of the fractions are also shown in Chart 2. The most active ethyl acetate fraction was fractionated repeatedly by silica gel column chromatography with a benzene–acetone or chloroform–methanol gradient system as the developer, and the fractions were monitored by thin layer chromatography (TLC, silica gel), high performance liquid chromatography (HPLC, LiChrosorb RP-8) and also by measurement of inhibitory activity against XO. The active fractions were purified by recrystallization. These active constituents were identified as genkwanin (**1**),[3,5–7)] apigenin (**2**),[3,6–9)] luteolin 7-methyl ether (**3**)[5)] and luteolin (**4**)[6,7,9,10)] on the bases of melting points, spectral comparisons and

elmental analysis. The concentrations of inhibitor in the assay mixture required to give 50% inhibition (IC_{50}) were as follows: **1**, $>10^{-5}$ M; **2**, 7.4×10^{-7} M; **3**, $>10^{-5}$ M; **4**, 5.9×10^{-7} M.

Kinetic studies were done on the effects of **2** and **4** on the oxidation of xanthine by XO under our assay conditions. The results are shown as Lineweaver–Burk plots[11] in Fig. 1. The mode of inhibition by **2** or **4** was of mixed type with respect to xanthine.

Beiler[12] and Westerfeld[13] investigated the inhibition of XO by flavonoids and related compounds and reported that 3,3′,4,4′-tetrahydroxychalcone and 3′,4′-dihydroxy-3,4-methylenedioxychalcone have strong inhibitory activities. Recently Otsuka, *et al.*[14] found a strong XO inhibitor, diosmetin, which is also a flavonoid.

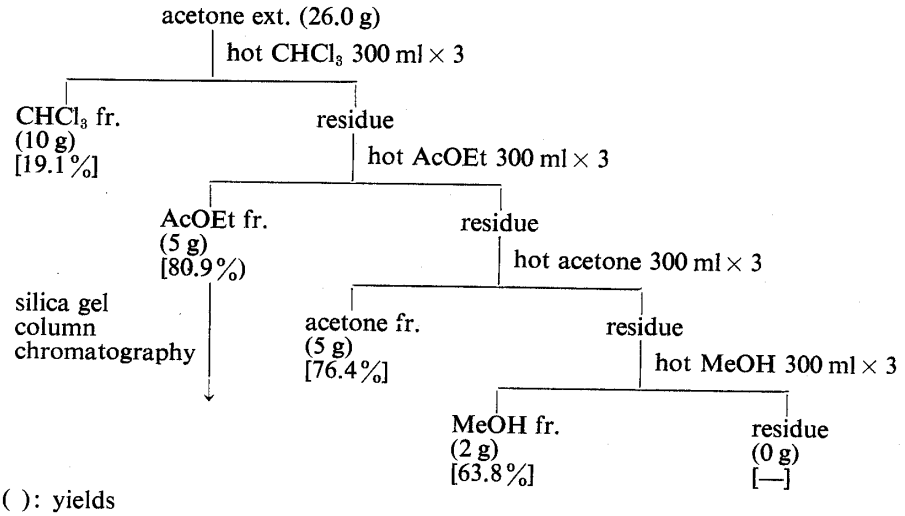
Here we report that **2** and **4**, constituents of the flowers and buds of *Daphne genkwa*, are strong inhibitors of XO under our assay conditions. On the other hand, these flavonoids, **1**, **2**, **3** and **4**, did not show strong inhibitory activities against monoamine oxidase under our assay



( ): yields

[ ]: percent inhibition (IC), 10 μg of test sample per 1 ml of reaction solution

Chart 1



( ): yields

[ ]: percent inhibition (IC), 10 μg of test sample per 1 ml of reaction solution

Chart 2

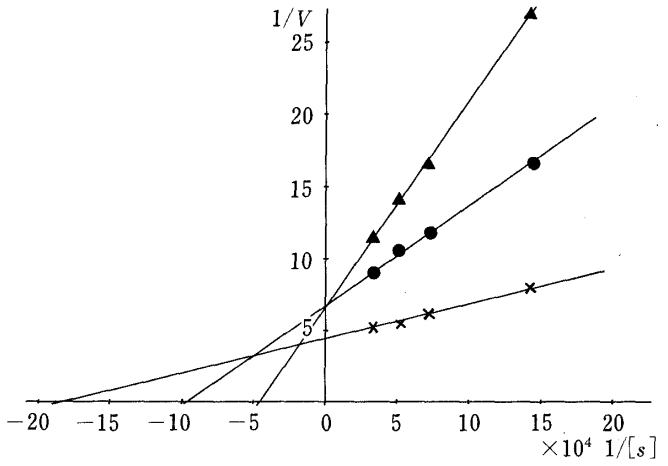


Fig. 1. Inhibitory Effects of Apigenin (**2**) and Luteolin (**4**) on XO

Lineweaver–Burk plots in the absence (0 M, ×—×) and in the presence of **2** (3.0×10^{-6} M, ○—○) or **4** (4.3×10^{-6} M, △—△) with xanthine as the substrate.
V, μmol substrate metabolized/mg enzyme/min; *s*, substrate.

conditions.[1] This is the first report of isolation of **3** and **4** from these flowers and buds.

Experimental

The following instruments were used to obtain physical data. Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. The infrared (IR) spectra were recorded on a JASCO IRA-202 infrared spectrophotometer. The ultraviolet (UV) spectra were recorded on a Shimadzu UV-360 recording spectrophotometer. The proton nuclear magnetic resonance (^{1}H-NMR) spectra and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a JEOL LNM-FX 90Q FT NMR spectrometer (90 MHz) with tetramethylsilane as an internal standard (δ value; s, singlet; d, doublet; t, triplet; q, quartet; br, broad). Mass spectra (MS) were recorded on a JEOL JMS-01SG-2 mass spectrometer. Silica gel 60 GF_{254} (Merck) was used for TLC and detection was achieved by illumination with an ultraviolet lamp, by spraying 3% $FeCl_3$ ethanol solution or by spraying 20% H_2SO_4 aq followed by heating. For column chromatography, Silica gel 60 (Merck) was used. HPLC was carried out by using a K-880 liquid chromatograph (Kyowa Seimitsu Co., Tokyo), combined with a JASCO UVIDEC-100 UV spectrophotometer and a LiChrosorb RP-8 (10 μm) column (250 × 4 mm). The eluent for HPLC was 5% aqueous formic acid–methanol (3 : 7) and the UV detector was set at 280 or 340 nm. The spectrophotometric measurements were carried out with a Hitachi model 101 spectrophotometer.

Enzyme and Chemicals——Xanthine oxidase (EC 1.2.3.2) from cow's milk was obtained from Boehringer Mannheim Co., Ltd. Xanthine was obtained from ICN Pharmaceuticals Inc. Tween 80 was obtained from Wako Pure Chemical Industries, Ltc. Sodium phosphate dibasic 12 hydrate and potassium phosphate monobasic were obtained from Kanto Chemical Co., Inc. The buffer used was Hasting–Sendroy's 1/15 M potassium phosphate–sodium phosphate buffer, pH 7.5. The substrate solution, 0.15 mM xanthine in water, was prepared immediately before use. Enzyme solution containing about 0.04 unit per ml in 1/15 M phosphate buffer, pH 7.5, was prepared immediately before use.

Test Solution——When the test samples were not easily soluble in water, they were dissolved in ethanol and Tween 80, and diluted with water to give final concentrations of 3% (v/v) for ethanol and 1.5% (w/v) for Tween 80 in the assay mixtures. At these concentrations of ethanol and Tween 80, XO activities were little affected.

Assay of Xanthine Oxidase Activity——The XO activities with xanthine as the substrate were measured spectrophotometrically by the method of Kalckar[4] with the following modification. The assay mixture consisted of 1.0 ml of test solution, 2.9 ml of 1/15 M phosphate buffer (pH 7.5) and 0.1 ml of enzyme solution. After preincubation of the mixture at 25 °C for 15 min, the reaction was initiated by adding 2.0 ml of substrate solution. This assay mixture was incubated at 25 °C for 30 min. The reaction was stopped by adding 1.0 ml of 1 N HCl, and the absorbance of the assay mixture at 290 nm was measured spectrophotometrically. A blank was prepared in the same way, but the enzyme solution was added to the assay mixture after adding 1 N HCl. One unit of XO was defined as the amount of enzyme producing 1 μmol of uric acid per min at 25 °C.

Estimation of Xanthine Oxidase Inhibitory Activity——XO inhibitory activity was expressed as the percentage inhibition of XO in the above assay system, calculated as $(1 - B/A) \times 100$, where A is the activity of the enzyme without test material and B is the activity of the enzyme with test material.

Extraction and Separation——The dried flowers and buds (1 kg, commercial products) of *Daphne genkwa* were extracted three times with 3 l of benzene under reflux for 3 h, then with $CHCl_3$, acetone, methanol and H_2O in the same way, and the extracts were concentrated *in vacuo*. These extracts were assayed for inhibitory effects against XO. The yield and inhibitory activities are shown in Chart 1. The most active acetone extract was extracted again with 300 ml of $CHCl_3$ under reflux for 30 min three times, then with AcOEt, acetone and MeOH in the same way. These fractions were concentrated *in vacuo* and were assayed for inhibitory effects against XO. The yields and inhibitory activities are shown in Chart 2. The most active AcOEt fr. was fractionated repeatedly by silica gel column chromatography with a benzene–acetone or $CHCl_3$–MeOH gradient system as the developer. The fractions were monitored by TLC, HPLC and also by measurements of inhibitory activities against XO. The active fractions were purified by recrystallization. Four active constituents were obtained, and were identified as genkwanin (**1**), apigenin (**2**), luteolin 7-methyl ether (**3**) and luteolin (**4**). The patterns of TLC and HPLC of AcOEt fr. and acetone fr. were similar, including flavonoids, **1**, **2**, **3** and **4**. About two-thirds of the MeOH fr. was a mixture of aglycones, **1**, **2**, **3** and **4**, and the other one-third was a mixture of glycosides of flavonoids. The percentages of the active flavonoids in the starting materials as determined by HPLC were as follows: **1** (0.22%), **2** (0.46%), **3** (0.34%) and **4** (0.15%).

Lineweaver–Burk Plots[11]——The Lineweaver–Burk plots for XO under our assay conditions were carried out in the absence and in the presence of **2** or **4** with xanthine as the substrate as shown in Fig. 1.

Genkwanin (1)——Yellow needles from acetone, mp 284—290 °C (lit. 286 °C).[3] *Anal.* Calcd for $C_{16}H_{12}O_5$: C, 67.60; H, 4.26. Found: C, 67.22; H, 4.22. This was identical with authentic genkwanin on the bases of mp, spectral comparisons[3,5-7] and elemental analysis.

Apigenin (2)——Yellow needles from acetone, mp 345—350 °C (lit. 352 °C).[3] *Anal.* Calcd for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73. Found: C, 66.38; H, 3.80. This was identical with authentic apigenin on the bases of mp, spectral comparisons[3,6-9] and elemental analysis.

Luteolin 7-Methyl Ether (3)——Yellow needles from MeOH, mp 286—289 °C (lit. 266—268 °C).[5] *Anal.* Calcd for $C_{16}H_{12}O_6$: C, 64.00; H, 4.03. Found: C, 63.87; H, 4.03. ^{13}C-NMR (DMSO-d_6) δ: 55.9 (q), 92.5 (d), 97.9 (d), 103.0 (d), 104.6 (s), 113.5 (d), 116.0 (d), 119.0 (d), 121.5 (s), 145.7 (s), 149.8 (s), 157.2 (s), 161.2 (s), 164.2 (s), 165.1 (s) and 181.7 (s). This was identical with authentic luteolin 7-methyl ether on the bases of spectral comparisons[5] and elemental analysis.

Luteolin (4)——Yellow needles from MeOH, mp 327—331 °C (lit. 327 °C).[10] *Anal.* Calcd for $C_{15}H_{10}O_6$: C, 62.94; H, 3.52. Found: C, 62.89; H, 4.03. This was identical with authentic luteolin on the bases of mp, spectral comparisons[6,7,9,10] and elemental analysis.

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References and Notes

1) This forms Part II of "Studies of Enzyme Inhibitors," Part I: T. Noro, T. Miyase, M. Kuroyanagi, A. Ueno and S. Fukushima, *Chem. Pharm. Bull.*, **31**, 2708 (1983). A part of this work was presented previously: Abstracts of Papers, the 103rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1983, p. 221.
2) R. W. Rundles, *Trans. Assoc. Am. Physicians*, **76**, 126 (1963); M. Isa, G. Hayashi and Y. Kowa, *Nippon Yakurigaku Zasshi*, **64**, 108 (1968).
3) M. Nakao and K. F. Tseng, *Yakugaku Zasshi*, **52**, 341, 903 (1932); R. Kasai, K. H. Lee and H. C. Huang, *Phytochemistry*, **20**, 2592 (1981).
4) H. M. Kalckar, *J. Biol. Chem.*, **167**, 429 (1947).
5) P. W. LeQuesne, S. B. Levery, M. D. Menachery, T. F. Brennan and R. F. Raffauf, *J. Chem. Soc., Perkin Trans. 1*, **1978**, 1572.
6) K. R. Markham, B. Ternai, R. Stanley, H. Geiger and T. J. Mabry, *Tetrahedron*, **34**, 1389 (1978).
7) H. Wagner, V. M. Chari and J. Sonnenbichler, *Tetrahedron Lett.*, **1976**, 1799.
8) H. Itokawa, K. Suto and K. Takeya, *Chem. Pharm. Bull*, **29**, 254 (1981).
9) T. Kurihara and M. Kikuchi, *Annual Report of Tohoku College of Pharmacy*, **20**, 35 (1973).
10) H. Nakamura and G. Fukuchi, *Yakugaku Zasshi*, **60**, 449 (1940).
11) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
12) J. M. Beiler and G. J. Martin, *J. Biol. Chem.*, **192**, 831 (1951).
13) W. W. Westerfeld and D. A. Richert, *J. Biol. Chem.*, **199**, 393 (1952).
14) K. Otsuka, Y. Ogata, H. Shiojiri, Y. Wakabayashi and K. Kagei, Abstracts of Papers, the 103rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1983, p. 221.