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Chalcones, coumarins, and flavanones from the exudate of *Angelica keiskei* and their chemopreventive effects

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Abstract

From an ethyl acetate-soluble fraction of the exudate obtained from the stems of *Angelica keiskei* (Umbelliferae), 17 compounds, viz. five chalcones (**1**–**5**), seven coumarins (**6**–**12**), three flavanones (**13**–**15**), one diacetylene (**16**), and one 5-alkylresorcinol (**17**), were isolated. These compounds were evaluated with respect to their inhibitory effects on the induction of Epstein-Barr virus early antigen (EBV-EA) by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells, which is known to be a primary screening test for antitumor-promoters. With the exception of three compounds (**10**, **16**, and **17**), all other compounds tested showed potent inhibitory effects on EBV-EA induction (92–100% inhibition at 1×10^3 mol ratio/TPA). In addition, upon evaluation of these compounds for the inhibitory effects against activation of (±)-(*E*)-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexemide (NOR 1), a nitric oxide (NO) donor, as a primary screening test for antitumor-initiators, two chalcones (**2** and **3**) and six coumarins (**6**-**11**) exhibited potent inhibitory effects.

Keywords: *Angelica keiskei*; Umbelliferae; Chalcones; Coumarins; Flavanones; Chemopreventive effects; Antitumor-promoter; Epstein-Barr virus; Antitumor-initiator

1. Introduction

Angelica keiskei KOIDZUMI (Japanese name 'Ashitaba', Umbelliferae) is a hardy perennial herb, growing mainly along the Pacific coast of Japan, that is traditionally used as a diuretic, laxative, analeptic and lactagogue [1]. The fresh leaves of this plant and its dry powder are used for food. Various chalcones [1–6], represented by xanthoangelol (**1**) and 4-hydroxyderricin (**4**), and coumarins [1,3,6] have so far been isolated and characterized from the plant. The two chalcones **1** and **4** have been proved to have antitumor-promoting activity in mouse

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skin carcinogenesis induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [6]. In this paper, we report the isolation and identification of 17 compounds from an ethyl acetate (EtOAc)-soluble fraction of the exudate of Ashitaba stems and inhibitory effects on the induction of Epstein-Barr virus early antigen (EBV-EA) by TPA and on activation of (±)-(*E*)-methyl-2-[(*E*)-hydroxy-imino]-5-nitro-6-methoxy-3-hexemide (NOR 1), a nitric oxide (NO) donor, of 17 compounds, **1**–**17**, evaluated in a preliminary screen for their potential cancer chemopreventive activities.

2. Materials and methods

2.1. Extraction and isolation

Lyophilized and powdered exudate (4.93 g) obtained from the stems of *Angelica keiskei* (Hachijo-type) [1], cultivated in the farm at Lombok island (Indonesia) and harvested in 2000, was partitioned with *n*-hexane-methanol (MeOH)–H_2O (95:95:10, v/v/v) to give a MeOH–H_2O soluble fraction (4.55 g) which was further partitioned between ethyl acetate (EtOAc) and H_2O (1:1, v/v). The EtOAc soluble fraction (0.93 g) was subjected to medium-pressure liquid chromatography (MPLC) on an ODS column (50 cm × 25 mm i.d.; eluting solvent, MeOH; flow rate, 3.0 ml/min) which yielded fractions A [357 mg; retention time (t_R) 29.6 min], B (439 mg; t_R 31.6 min), and C (74 mg, t_R 40.4 min). Upon preparative HPLC on an ODS column (25 cm × 10 mm i.d.; eluting solvent, MeOH–H_2O–acetic acid, 65:35:1, v/v/v; flow rate, 2.0 ml/min), fraction A gave xanthoangelol (**1**) [4] (5.4 mg), isobavachalcone (**3**) [7] (8.6 mg), xanthoangelol H (**5**) [5] (1.4 mg), laserpitin (**6**) [8] (31.2 mg), isolaserpitin (**7**) [8] (15.6 mg), 3′-senecioyl khellactone (**8**) [8] (1.9 mg), 4′-senecioyl khellactone (**9**) [8] (3.7 mg), selinidin (**10**) [8] (6.8 mg), pteryxin (**11**) [9] (2.6 mg), (3′*R*)-3′-hydroxycolumbianidin (**12**) [10] (2.5 mg), mundulea flavanone A (**13**) [11] (1.7 mg), prostratol F (**14**) [7] (7.4 mg), and falcarindiol (**16**) [12] (14.0 mg) (see Fig. 1 for the structure); fraction B gave **1** (121.7 mg), **3** (1.3 mg), 4-hydroxyderricin (**4**) [4] (70.8 mg), **6** (3.8 mg), **7** (10.7 mg), **10** (15.1 mg), **13** (4.0 mg), **14** (1.0 mg), and **15** (1.7 mg); and fraction C yielded xanthoangelol F (**2**) [5] (35.0 mg), **4** (2.0 mg), 4′-*O*-geranylnaringenin (**15**) [13] (3.2 mg), and 5-*n*-pentadecylresorcinol (**17**) [14] (2.6 mg). Identification of all of the above compounds was performed by MS and ^{1}H NMR comparison with the literature.

2.2. Chemicals

The cell culture reagents and *n*-butyric acid from Nacalai Tesque, Inc. (Kyoto, Japan), TPA, β-carotene, and glyzyrrhizin from Sigma Co., NOR 1 and carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt] from Dojindo Laboratories (Kumamoto, Japan), were purchased.

2.3. In vitro EBV-EA induction tests

The EBV genome-carrying lymphoblastoid cells, Raji cells, derived from Burkitt's lymphoma, were cultured in RPMI-1640 medium. The Raji cells were incubated for 48 h at 37 °C in a medium containing *n*-butyric acid (4 mM), TPA (32 pM), and various amounts of test compounds. Smears were made from the cell suspension, and the EBV-EA-inducing cells were stained by means of an indirect immunofluorescence technique. The details of the in vitro assay on EBV-EA induction have been reported previously [15].

2.4. In vitro NOR 1 inhibition tests [16]

Chang liver cells (normal human hepato cells; 5×10^5/ml), derived from human liver in MEM Eagle medium, were cultured three days before treatment. NOR 1 was added into culture dish and incubated for 1 h under CO_2 incubator as control. For screening assay, test samples to culture dish were added before 1 min of NOR 1 treatment. Transformed cells were observed under light-microscopy (× 100). All observed cells count for more than 250. The inhibitory ratio was then calculated:

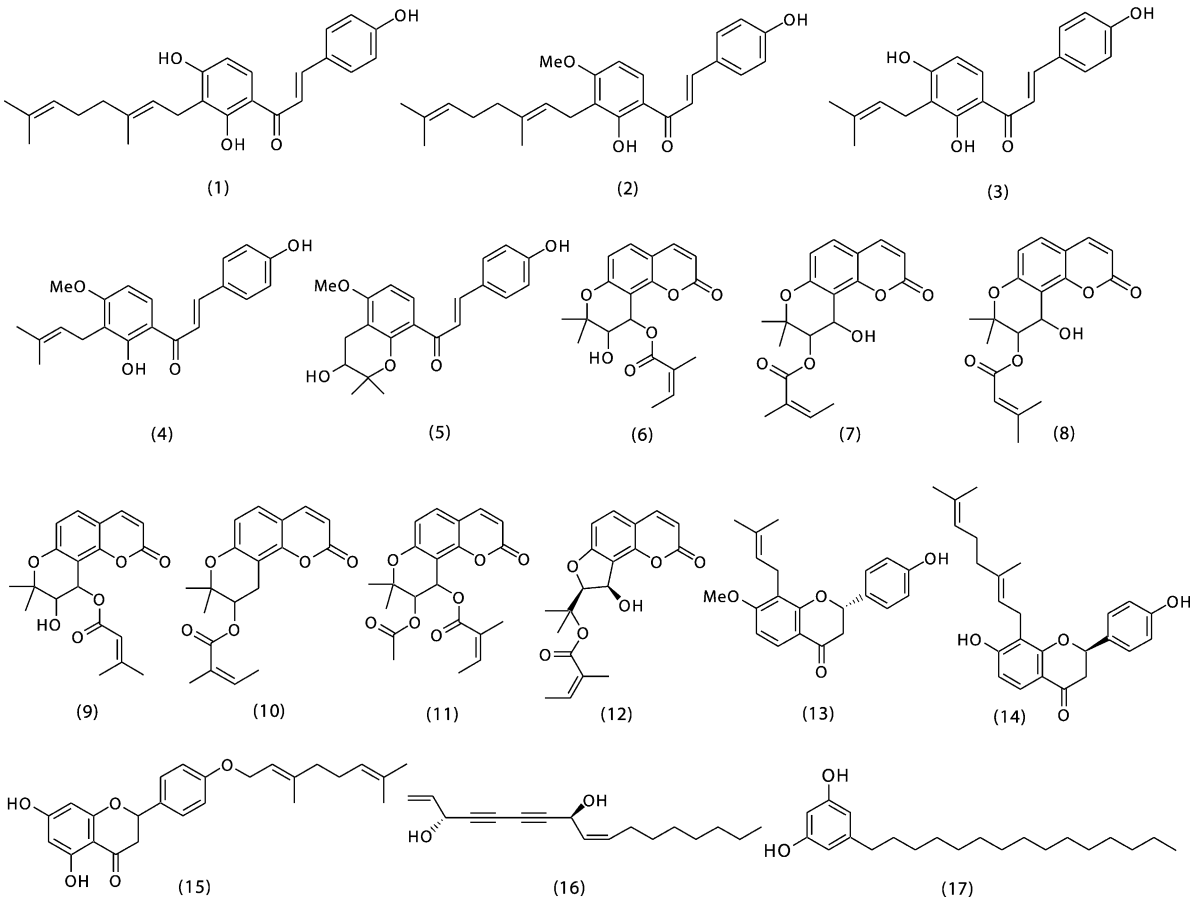

Fig. 1. Structures of compounds isolated from the exudate of *Angelica keiskei* stems.

Inhibitory ratio (IR)

$$= \frac{\text{transformed cell\% (NOR 1 alone)}}{\text{transformed cell\% (NOR 1 + test sample)}}$$

3. Results and discussion

In this study, we were able to isolate five chalcones (**1**–**5**), seven coumarins (**6**–**12**), three flavanones (**13**–**15**), one diacetylene (**16**), and one 5-alkylresorcinol (**17**) from an EtOAc-soluble fraction of the exudate obtained from Ashitaba stems. The inhibitory effects on the induction of EBV-EA induced by TPA were examined as a preliminary evaluation of the potent antitumor-promoting activities for these compounds. The inhibitory effects (Table 1) were compared with those of reference compound, β-carotene, a vitamin A precursor that has been studied intensively in cancer chemoprevention using animal models [17]. With the exception of three compounds (**10**, **16**, and **17**), all other compounds tested exhibited potent inhibitory effects (92–100% inhibition at 1×10^3 mol ratio, and 51–84% inhibition at 5×10^2 mol ratio), and among which two chalcones, **3** and **4**, and three flavanones, **13**–**15**, exhibited remarkably high inhibitory effects (100% inhibition at 1×10^3 mol ratio), on EBV-EA induction by TPA with preservation of the high viability (60–70%) of the Raji cells. The inhibitory effects of these compounds were equivalent to or more potent than β-carotene. Since two charconnes, **1** and **4**, have

Table 1
Inhibitory effects on the induction of Epstein-Barr Virus Early Antigen and Inhibitory Ratio (IR) on NOR 1 action of compounds **1**–**17** isolated from *Angelica keiskei* and reference compounds

Compound		Percentage of EBV-EA induction[a]					IR of NOR 1 activation[b]
		Concentration (mol ratio/TPA)					
		1000		500	100	10	
Chalcone							
1	Xanthoangelol	1.8	(60)	19.7	65.0	90.0	1.9
2	Xanthoangelol F	2.1	(60)	21.6	68.3	91.0	2.4
3	Isobavachalcone	0	(60)	16.3	63.0	88.4	2.0
4	4-Hydroxyderricin	0	(60)	17.3	64.1	93.2	1.8
5	Xanthoangelol H	5.3	(60)	32.4	71.5	95.0	1.5
Courmarin							
6	Lasepitin	0.3	(60)	48.9	78.3	100	2.0
7	Isolaserpitin	8.5	(60)	46.7	76.2	98.4	2.1
8	3′-Senecioyl khellactone	7.1	(60)	45.3	74.4	96.3	2.1
9	4′-Senecioyl khellactone	7.1	(60)	42.3	72.5	94.0	2.1
10	Selinidin	19.5	(60)	47.7	77.2	100	2.0
11	Pteryxin	4.3	(60)	41.0	72.4	93.2	2.2
12	(3′R)-3′-Hydroxycolumbianadin	3.5	(60)	40.3	71.7	93.0	1.5
Flavanone							
13	Munduleaflavanone A	0	(70)	25.2	69.3	90.3	1.7
14	Prostratol F	0	(70)	28.6	70.6	92.1	1.9
15	4′-*O*-Geranylnaringenin	0	(60)	20.3	73.1	93.2	1.9
Others							
16	Falcarindiol	23.1	(60)	56.3	85.5	100	1.5
17	5-*n*-Pentadecylresorcinol	12.4	(60)	45.9	74.1	100	1.8
Reference compound							
	β-Carotene	8.6	(70)	34.2	82.1	100	
	Glyzyrrhizin	26.4	(>80)	63.3	83.3	100	2.2
	Carboxy-PTIO						8.0

[a] Values represent percentages relative to the positive control value. TPA (3 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells.
[b] Determined at the concentration of 350 nmol. Inhibitory ratio of NOR 1 (positive control; 350 nmol) was taken as 1.0.

already been proved to have antitumor-promoting activity in mouse skin carcinogenesis using DMBA as an initiator and TPA as a promoter [6], and since the inhibitory effects against EBV-EA induction have been demonstrated to closely parallel those against tumor promotion in vivo [15], the three other chalcones (**2**, **3**, **5**), six coumarins (**6**–**9, 11**, **12**), and three flavanones (**13**–**15**), also are suggested to be valuable antitumor-promoters. Details of the specificity of EBV-EA induction test were described previously [18].

Using in vitro screening models for nitric oxide (NO) scavenging [16], 17 compounds, **1**–**17**, from Ashitaba exudate were evaluated for their scavenging activity against NO generation by NOR 1 in cultured cell system. Table 1 showed the inhibitory ratios (IR) of the compounds and two reference compounds: a natural compound, glycyrrhizin, and a synthetic NO scavenger, carboxy-PTIO, on NOR 1 action. Among the compunds tested, two chalcones, **2** and **3**, and six coumarins, **6**–**11**, exhibited potent inhibitory effects (IR = 2.0–2.4) which were almost equivalent to that of glyzyrrhizin (IR = 2.2).

From the results of in vitro EBV-EA induction and in vitro NOR 1 inhibition tests, it might be suggested that chalcones, coumarins, and flavanones from the exudate of Ashitaba stems to be useful as

chemopreventive agents in chemical carcingenesis, and the exudate of Ashitaba stems are valuable food ingredient by containing these compounds abundantly.

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References

[1] K. Baba, M. Taniguchi, K. Nakata, Studies on *Angelica keiskei* ashitaba, Foods Food Ingredients J. Jpn. 178 (1998) 52–60.

[2] M. Kozawa, N. Morita, K. Baba, H. Hata, Chemical components of the roots of *Angelica keiskei* KOIDZUMI. II. The structure of chalcone derivatives, Yakugaku Zasshi 98 (1978) 210–214.

[3] K. Baba, T. Kido, Y. Yoneda, M. Taniguchi, M. Kozawa, Chemical components of *Angelika keiskei* KOIDZUMI (V) Components of the fruits, and comparison of coumarins and chalcones in the fruits, roots and the leaves, Syoyakugaku Zasshi 44 (1990) 235–239.

[4] K. Baba, K. Nakata, M. Taniguchi, T. Kido, M. Kozawa, Chalcones from *Angelica keiskei*, Phytochemistry 29 (1990) 3907–3910.

[5] K. Nakata, M. Taniguchi, K. Baba, Three chalcones from *Angelica keiskei*, Nat. Med. 53 (1999) 329–332.

[6] T. Okuyama, M. Takata, J. Takayasu, T. Hasegawa, H. Tokuda, A. Nishino, H. Nishino, A. Iwashima, Anti-tumor-promotion by principles obtained from *Angelica keiskei*, Planta Med. 57 (1991) 242–246.

[7] M. Iinuma, M. Ohyama, T. Tanaka, Two flavanones from roots of *Sophora leachiana*, Phytochemistry 38 (1995) 539–543.

[8] T.M. Swager, J.H. Cardellina II, Coumarins from *Musineon divaricatum*, Phytochemistry 24 (1985) 805–813.

[9] I.S. Chen, C.T. Chang, W.S. Sheen, C.M. Teng, I.L. Tsai, C.Y. Duh, F.N. Ko, Coumarins and antiplatelet aggregation constituents from Formosan *Peucedanum japonicum*, Phytochemistry 41 (1996) 525–530.

[10] K. Baba, T. Hamasaki, Y. Tabata, M. Kozawa, Y. Honda, M. Tabata, Chemical studies on Chinese crude drug 'She Chuang Zi', Syoyakugaku Zasshi 39 (1985) 282–290.

[11] E. Venkata Rao, P. Sridhar, R. Prasad, Two prenylated flavanones from *Mundulea suberosa*, Phytochemistry 46 (1997) 1221–1274.

[12] A. Satoh, Y. Narita, N. Endo, H. Nishimura, Potent allelochemical falcalindiol from *Glehnia littoralis* F. Schm, Biosci. Biotech. Biochem. 60 (1996) 152–153.

[13] M. Ahsan, J.A. Armstrong, S. Gibbons, A.I. Gray, P.G. Waterman, Novel *O*-prenylated flavonoids from two varieties of *Boronia coerulescens*, Phytochemistry 37 (1994) 259–266.

[14] D.D. Ridley, E. Ritchie, W.C. Taylor, Chemical studies of the Proteaceae II. Some further constituents of *Grevillea robusta* A. Cunn.; Experiments on the synthesis of 5-*n*-tridecylresorcinol (grevillol) and related substances, Aust. J. Chem. 21 (1968) 2079–2988.

[15] Y. Takaishi, K. Ujita, H. Tokuda, H. Nishino, A. Iwashima, T. Fujita, Inhibitory effects of dihydroagarofuran sesquiterpenes on Epstein-Barr virus activation, Cancer Lett. 65 (1992) 19–26.

[16] H. Tokuda, M. Kuchide, M. Okuda, T. Mukainaka, Y. Nobukuni, J. Takayasu, H. Nishino, Chemopreventive activity of antioxidants on nitric oxide donors induced mouse skin carcinogenesis, Proc. Am. Assoc. Cancer Res. #5674 (2002) 1145.

[17] A. Murakami, H. Ohigashi, K. Koshimizu, Anti-tumor promotion with food phytochemicals: A strategy for cancer chemoprevention, Biosci. Biotech. Biochem. 60 (1996) 1–8.

[18] Y. Ito, H. Tokuda, H. Ohigashi, K. Koshimizu, Distribution and characterization of environmental promoter substances as assay by synergistic Epstein-Barr virus-activating system, in: H. Fujiki, et al. (Eds.), Cellular Interactions by Environmental Tumor Promoters, VNU Science Press, Utrecht, 1984, pp. 125–137.