Anti-tumor Promoting Effect of Glycosides from *Prunus persica* Seeds

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Four minor components, along with the major cyanogenic glycosides, amygdalin and prunasin, were isolated from *Prunus persica* seeds (Persicae Semen; Tounin), and characterized as mandelic acid glycosides (β-gentiobioside and β-D-glucoside) and benzyl alcohol glycosides (β-gentiobioside and β-D-glucoside). The anti-tumor promoting activity of these compounds was examined in both *in vitro* and *in vivo* assays. All of the compounds significantly inhibited the Epstein-Barr virus early antigen activation induced by tumor promoter. In addition, they produced a delay of two-stage carcinogenesis on mouse skin that was comparable in potency to (−)-epigallocatechin gallate from green tea. Structure–activity relationships indicated that a substituent at the benzylic position with glycosidic linkage affected the *in vitro* and *in vivo* activities with an order of enhancing potency, CN<COOH<H.

Key words *Prunus persica* seed; cancer chemoprevention; amygdalin-related glycoside; benzyl β-gentiobioside; EBV-EA induction; mouse skin two-stage carcinogenesis

Prunus persica (L.) BATSCH (Rosaceae) seeds are well known as a traditional medicine (Persicae Semen; Tounin, Taoren in Chinese) in Japan, China, and other Asian countries. They are frequently used as an ingredient in a variety of Kampo (Chinese medicine) prescriptions, particularly those used to treat women's diseases. The chemical constituents of the herb include the cyanogenic glycosides, amygdalin (**1**) and prunasin (**2**) as major components,[1] along with glycerides,[2,3] sterols,[4] and emulsin.[5] Amygdalin is also abundant in the seeds of bitter almond and apricots of the *Prunus* genus, and other rosaceous plants.[6] Amygdalin (**1**), sometimes referred to as vitamin B_{17}, and nitriloside was previously thought to be synonymous with laetrile, a drug used to treat cancer.[7] However, studies now show that laetrile, an acronym for laevorotatory nitriloside, is ineffective as a cancer treatment, and is structurally different from amygdalin.[8] Thus, the antitumor effect of **1** remains ambiguous. In our phytochemical investigation of Persicae Semen, we isolated and characterized four minor components that were structurally related to **1** and **2**, and examined their function and antitumor properties. Here we report the inhibitory effect of purified constituents from Persicae Semen on the induction of Epstein-Barr virus early antigen (EBV-EA) in Raji cells,[9] a convenient *in vitro* assay for assessing anti-tumor promoting activity. *In vivo* anti-tumor promoting activity was also evaluated using two-stage mouse skin carcinogenesis test.

MATERIALS AND METHODS

Isolation and Characterization of Minor Constituents Persicae Semen (500 g), purchased from Uchida Wakanyaku Co., Ltd., Tokyo, was homogenized in 70% aqueous ethanol (5 l). The concentrated homogenate was extracted with *n*-hexane (3 l), ethyl acetate (3 l) and 1-butanol (3 l), successively. The 1-butanol extract (5.0 g) was chromatographed over a porous polymer gel, Diaion HP-20 (Mitsubishi Chemical Co.) with H_2O, 10% ethyl alcohol (EtOH), 20% EtOH and EtOH. Eluates with 10% EtOH (0.98 g) and 20% EtOH (0.48 g) were used for preparative HPLC [Waters μ-Bondasphere C-18 (5 μ) 100 Å, i.d. 19×150 mm; CH_3CN–H_2O (13 : 87, 10 : 90 or 5 : 95) or MeOH–H_2O (15 : 85)], and six constituents, **1** (671.8 mg), **2** (173.7 mg), **3** (32.5 mg), **4** (37.0 mg), **5** (74.4 mg), and **6** (13.8 mg), were isolated. Four minor constituents (**3** to **6**) were identified by comparison of their physicochemical data with those reported in the literature as amygdalinic acid (mandelic acid β-gentiobioside) (**3**),[8] [high resolution (HR)-MS: *m/z* 499.1451 $(M+Na)^+$: Calcd for $C_{20}H_{28}O_{13}$+Na, 499.1428, $[\alpha]_D$ −108° (*c*=0.6, MeOH)], mandelic acid β-D-glucopyranoside (**4**)[10] [HR-MS: *m/z* 337.0899 $(M+Na)^+$: Calcd for $C_{14}H_{18}O_8$+Na, 337.0899, $[\alpha]_D$ −83° (*c*=0.5, C_5H_5N)], benzyl β-gentiobioside (**5**)[11] [HR-MS: *m/z* 450.1932 $(M+NH_4)^+$: Calcd for $C_{19}H_{28}O_{11}$+NH_4, 450.1975, $[\alpha]_D$ −58.3° (*c*=1.0, MeOH)], and benzyl β-D-glucopyranoside (**6**)[12] [HR-MS: *m/z* 288.1475 $(M+NH_4)^+$: Calcd for $C_{13}H_{18}O_6$+NH_4, 288.1447. $[\alpha]_D$ −56° (*c*=0.5, MeOH)], respectively.

Assay for Inhibition of EBV-EA Activation EBV-EA-positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medical University. EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui). Spontaneous EBV-EA activation in our Raji cell subline was less than 0.1%. Inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type) as described previously.[9,12] Indicator cells (Raji, 1×10^6 cells/ml) were incubated at 37 °C for 48 in 1 ml of medium containing 0.5 M *n*-butyric acid [8 μl, 4 mmol (co-inducer)] and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [32 pmol=20 ng in dimethylsulfoxide (DMSO), 2 μl] as an

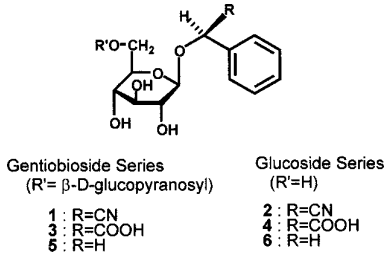

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Table 1. Effect of Glycosides from *P. persica* Seeds on TPA-Induced EBV-EA Activation

	Concentration (mol ratio/TPA)			
	1000	500	100	10
	% relative to the positive control			
Amygdalin (**1**)	13.8±0.6 (60)	40.4±1.2 (>80)	67.9±1.7 (>80)	100±0.1 (>80)
Prunasin (**2**)	5.6±0.3 (70)	52.7±1.3 (>80)	80.2±2.1 (>80)	100±0.3 (>80)
Amygdalinic acid (**3**)	3.5±0.3 (60)	34.1±1.2 (>80)	65.0±1.9 (>80)	95.7±0.4 (>80)
Mandelic acid β-D-glucopyranoside (**4**)	11.2±0.5 (70)	35.7±1.4 (>80)	67.4±2.0 (>80)	100±0.1 (>80)
Benzyl β-gentiobioside (**5**)	0±0.2 (60)	20.5±1.1 (>80)	48.3±1.3 (>80)	90.6±0.3 (>80)
Benzyl β-D-glucopyranoside (**6**)	4.8±0.4 (70)	28.0±1.3 (>80)	55.9±1.9 (>80)	96.4±0.3 (>80)
(−)-Epigallocatechin gallate	6.4±0.3 (70)	34.9±1.3 (>80)	68.1±1.7 (>80)	87.7±0.3 (>80)

Values in parentheses are the viability percentage of Raji cells.

inducer, with or without various amounts of test compounds in 5 μl DMSO. Smears were made from the cell suspension, and activated cells that were stained by EBV-EA-positive serum from the NPC patient were detected by an indirect immunofluorescence technique.[14] At least 500 cells were counted in each assay, and the number of stained (positive) cells was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compounds was expressed as the relative ratio to the control experiment (100%), which was carried out with only *n*-butyric acid (4 mmol) plus TPA (32 pmol). EBV-EA induction was typically around 35%. The viability of treated Raji cells was assayed by Trypan Blue staining.

Assay for Anti-tumor Promoting Activity in Two-Stage Mouse Skin Carcinogenesis Assays were performed according to a previously described method.[15] Specific pathogen-free female ICR mice (six weeks old) were obtained from Japan SLC Inc., Shizuoka, Japan. The animals were housed, five per polycarbonate cage, in a temperature-controlled room at 24±2 °C and given food and water *ad libitum* throughout the experiment. Animals were divided into three experimental groups containing 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with 7,12-dimethylbenz[a]anthracene (DMBA) (100 μg, 390 nmol) in acetone (0.1 ml) as an initiating treatment. One week after initiation, papilloma formation was promoted twice weekly by applying 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (1 μg, 1.7 nmol) in acetone (0.1 ml) to the skin. One hour before each treatment, mice were treated with the samples (85 nmol) in acetone (0.1 ml). Papilloma incidence was examined weekly over a period of 20 weeks.

RESULTS AND DISCUSSION

Besides the major cyanogenetic glycosides **1** and **2**, we found four minor glycosides in Persicae Semen, characterized as amygdalinic acid (**3**), mandelic acid β-D-glucopyranoside (**4**), benzyl β-gentiobioside (**5**) and benzyl β-D-glucopyranoside (**6**). Of these compounds, **6** has also been found in *P. mume* fruits, and was reported to have hypotensive diuretic properties.[16] In addition, compound **3** was reported to have anticancer property upon oral administration to mice having inoculated tumor cells (AC755).[17] On the other hand, Okuyama *et al.* reported that Kampo prescriptions such as Toukaku-johki-to (Taone-chengqi Tang) including Persicae Semen as an ingredient, which are employed for an Oketsu syndrome (stagnation of blood circulations), showed a relatively strong anti-tumor promoting effect.[18] These findings may imply that Persicae semen contributes to the effect. As a part of investigation to develop the function of the Persicae Semen constituents, we examined their suppressive effects on the tumor-promotion stage during multi-stage chemical carcinogenesis.

The inhibitory TPA-induced EBV-EA activation test in Raji cells was examined first for **1**—**6**, and the results are shown in Table 1. Amygdalinic acid (**3**), benzyl β-gentiobioside (**5**) and benzyl β-D-glucopyranoside (**6**) produced remarkable inhibition (65—80%) of EBV-EA activation at a concentration of 500 mol ratio/TPA, without exhibiting cytotoxicity. Their potencies were either comparable to, or much stronger than the positive control, (−)-epigallocatechin gallate (EGCG), which is a well known anti-tumor promoting polyphenol from green tea.[19] In both the gentiobiosides and the glucosides, the inhibitory effect was dependent on a substitutent at the benzylic position of the aglycone with an order of potency, CN<COOH<H. Gentiobiosides showed stronger activity than the glucoside series with the same substituent, *i.e.*, **1**>**2**, **3**>**4**, and **5**>**6**.

The isolated compounds were then subjected to an *in vivo* two-stage mouse skin carcinogenesis assay using DMBA as an initiator and TPA as a promoter. The results are shown in Fig. 1. The control animals exhibited a 100% papilloma incidence 10 weeks after initiation. However, treatment with the tested compounds (85 nmol), along with initiator and promoter, reduced the percentage of tumor-bearing mice by between 13.3—33.3% after 10 weeks (Fig. 1A). Structure–activity relationships in this assay were similar to those in the *in vitro* EBV-EA activation assay, although the *in vivo* test for **6** could not be examined owing to insufficient amounts available. Thus, benzyl β-gentiobioside (**5**) had the most potent activity, reducing the incidence to 86.6% over 20 weeks. In the treated animals, the average number of papillomas per mouse was reduced to about 44.0% to 59.3% relative to the control group by week 20 (Fig. 1B). It is noteworthy that the potency of **5** and **3** was almost comparable to that of EGCG at the same concentration (85 nmol) (Fig. 1).

Chemoprevention is an efficient strategy for cancer prevention, and extensive efforts have been made in recent years to find natural and synthetic products that prevent the tumor-promoting stage, which is a long and reversible process during multi-stage carcinogenesis in humans. In the present

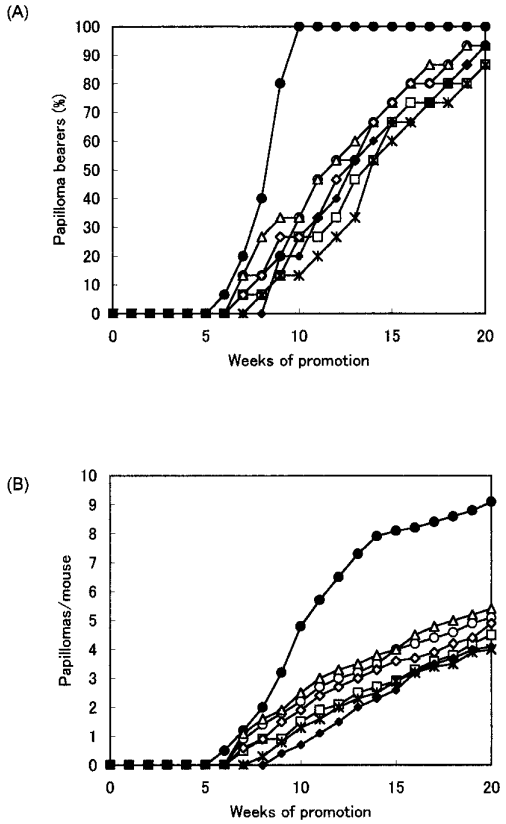

Fig. 1. Inhibition of TPA-Induced Tumor Promotion by Multiple Applications of Glycosides (Each 85 nmol) from Persicae Semen

(A) Percentage of mice bearing papillomas. (B) Average number of papillomas per mouse. ● control (TPA alone); ○ TPA+1; △ TPA+2; □ TPA+3; ◇ TPA+4; ＊TPA+5; ◆ TPA+EGCG.

study, some structurally simple glycosides were found to have significant anti-tumor promoting activity. It should be noted that amygdalinic acid (**3**) might be effective agent not only for inhibition of the growth of tumors (AC755) inoculated in mice[7] but also for chemoprevention of cancer. These compounds, as well as the Persicae Semen extract, would be a promising source of possible cancer chemopreventive agents, and valuable to be further studied.

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