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Cancer preventive agents. Part 8: Chemopreventive effects of stevioside and related compounds

Midori Takasaki [a], Takao Konoshima [a], Mutsuo Kozuka [b], Harukuni Tokuda [c], Junko Takayasu [c], Hoyoku Nishino [c], Masazumi Miyakoshi [d], Kenji Mizutani [d], Kuo-Hsiung Lee [b,*]

[a] Faculty of Pharmaceutical Sciences, Chiba Institute of Science, Choshi, Chiba 288-0025, Japan
[b] Natural Products Research Laboratories, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA
[c] Department of Molecular Biochemistry, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto, 602-0841, Japan
[d] Research Center, Maruzen Pharmaceutical Co., Ltd., Fukuyama. Hiroshima, 729-3102, Japan

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ABSTRACT

In a search for potential cancer chemopreventive agents from natural resources, stevioside (**1**), a sweetener, and six related compounds, including two aglycones steviol (**6**) and isosteviol (**7**), were screened in an in vitro assay for inhibitory effects on Epstein–Barr virus early antigen activation. Compounds **1**, **6** and **7** showed significant activity in this assay and also exhibited strong inhibitory effects in a two-stage carcinogenesis test using mouse skin induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The inhibitory effects of these three compounds were greater than that of glycyrrhizin. Furthermore, these three compounds significantly inhibited mouse skin carcinogenesis initiated by peroxynitrite and promoted by TPA. Their activities were comparable to that of curcumin. These results suggested that **1**, as well as **6** and **7**, could be valuable as chemopreventive agents for chemical carcinogenesis.

1. Introduction

Cancer is a major cause of death worldwide and causes serious problems in human life, including mental and physical agony and economic strain. Therefore, many kinds of cancer therapies, including various anticancer agents, have been developed. However, they also have several problems such as serious side effects and drug resistance. To resolve these difficulties, development of cancer chemopreventive agents and improvement of cancer treatment are very important. Among the many stages of chemical carcinogenesis, inhibition of the tumor promotion stage has been regarded as one of the most promising methods for chemoprevention. It has also been ascertained that overproduced nitric oxide (NO) is changed to peroxynitrite, which can induce damage on genes, cells, and tissues that can lead to mutagenesis and carcinogenesis. Such experimental proof verifies that NO and peroxynitrite can strongly initiate multi-stage carcinogenesis.[1]

With these facts in mind, we have been studying cancer chemopreventive agents from natural products, and determining their antitumor-promoting and antitumor-initiating effects.[2–6] In continuation of these studies, diterpenoids and triterpenoids were examined using both an in vitro primary screening test for inhibition of Epstein–Barr virus early antigen (EBV-EA) activation promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and an in vivo two-stage carcinogenesis test using 7,12-dimethylbenz[*a*]anthracene (DMBA) or peroxynitrite as an initiator and TPA.[7–10] In this paper, we report the inhibitory effects of the natural sweetener stevioside (**1**)[11] from *Stevia rabaudiana* and its two aglycones, steviol (**6**)[12] and isosteviol (**7**),[12,13] obtained by enzymatic hydrolysis and by acidic hydrolysis, respectively, on mouse skin carcinogenesis and their probability for use as cancer chemopreventive agents in chemical carcinogenesis.

2. Results and discussion

2.1. Inhibition of EBV-EA activation assay

A primary screening test was carried out using a short-term in vitro synergistic assay on EBV-EA activation. Table 1 lists inhibitory effects of five sweet diterpenoid glycosides (**1**–**5**) obtained from the leaves of *Stevia rabaudiana* Bertoni and their two hydrolytic products steviol (**6**), genuine aglycone, and isosteviol (**7**), artifact aglycone, on the EBV-EA activation induced by TPA and the associated viability of Raji cells.[11,12,15]

In this assay, all compounds tested showed inhibitory effects on EBV-EA activation without cytotoxicity on Raji cells. As shown in Table 1, among the diterpenoid glycosides, stevioside (**1**) exhibited

* Corresponding author. Tel.: +1 919 962 0066; fax: +1 919 966 3893?
E-mail address: khlee@unc.edu (K.-H. Lee).

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Table 1
Relative ratio of EBV-EA activation with respect to positive control (100%) in the presence of stevioside-related compounds (**1**–**7**)

Compound	Concentration[a]				IC_{50} (μM)
	1000	500	100	10	
Stevioside (**1**)	3.2[b] ± 0.7 (60)[c]	36.9 ± 0.6 (>80)	67.4 ± 0.8 (>80)	89.3 ± 0.5 (>80)	360
Rebaudioside A (**2**)	14.8 ± 0.6 (60)	47.3 ± 0.6 (>80)	82.5 ± 0.9 (>80)	100.0 ± 0.5 (>80)	463
Rebaudioside C (**3**)	15.4 ± 0.6 (60)	46.9 ± 0.6 (>80)	83.7 ± 0.9 (>80)	100.0 ± 0.5 (>80)	469
Dulcoside A (**4**)	6.7 ± 0.6 (60)	40.9 ± 0.6 (>80)	73.0 ± 0.8 (>80)	92.5 ± 0.5 (>80)	447
Rubusoside (**5**)	12.5 ± 0.6 (60)	44.2 ± 0.6 (>80)	76.8 ± 1.3 (>80)	94.2 ± 0.5 (>80)	450
Steviol (**6**)	2.1 ± 0.3 (60)	35.6 ± 0.6 (>80)	74.1 ± 1.1 (>80)	91.2 ± 0.5 (>80)	340
Isosteviol (**7**)	1.9 ± 0.2 (60)	34.2 ± 0.5 (>80)	73.0 ± 1.1 (>80)	90.6 ± 0.3 (>80)	321
Glycyrrhizin (**8**)	12.5 ± 1.0 (60)	36.9 ± 1.2 (>80)	69.2 ± 2.1 (>80)	100.0 ± 0.4 (>80)	381
Curcumin (**9**)	0 ± 0.5 (60)	22.8 ± 1.8 (>80)	81.7 ± 1.9 (>80)	100 ± 0.2 (>80)	331

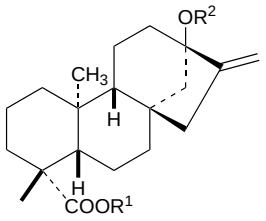
[a] Mol ratio/TPA (32 pmol/mL = 20 ng/mL).
[b] Values represent relative percentage to the positive control (100%) (n = 3 and ±SD).
[c] Values in parentheses are viability percentages of Raji cells.

the strongest inhibitory effects on EBV-EA activation (IC_{50} = 360 μM, with >96%, 63%, and 32% inhibition at 1×10^3, 5×10^2, 1×10^2 mol ratio/TPA, respectively), and preserved a high viability of Raji cells. Steviol (**6**) and isosteviol (**7**) showed almost the same inhibitory effects (for **6**: IC_{50} = 340 μM, with >97%, 64%, and 25% inhibition; for **7**: IC_{50} = 331 μM, with 98%, 65%, and >26% inhibition at 1×10^3, 5×10^2, 1×10^2 mol ratio/TPA). [Previously, Okamoto et al. reported that **6** inhibited the activation of EBV-EA induced by TPA, while **1** did not.[16] Also, Akihisa et al. reported inhibitory effects of **1** and **7** on activation of EBV-EA induced by TPA.[17]]

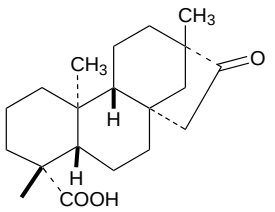
In our experiments, the inhibitory effects of stevioside (**1**), steviol (**6**), and isosteviol (**7**) were greater than that of glycyrrhizin (**8**), which is a known antitumor-promoting agent.[18] In our past work, inhibitory effects on EBV-EA activation by TPA correlated well with antitumor-promoting activity in vivo.[2–10] Thus, on the basis of the results of the in vitro assay, the inhibitory effects of these three compounds were investigated in a two-stage carcinogenesis test of mouse skin tumors using DMBA as an inhibitor and TPA as a promoter.

2.2. Two-stage carcinogenesis test on mouse skin induced by DMBA and TPA

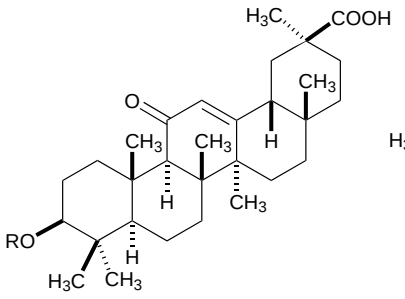
Figures 2 and 3 show the time course of tumor formation after treatment with **1** and the aglycones **6** and **7**, respectively. The incidence (%) of papilloma-bearing mice and the average numbers of papillomas per mouse are presented in Figures 2A/3A and Figures 2B/3B, respectively.



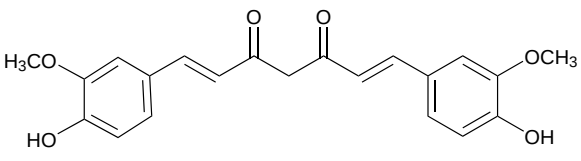
1: stevioside R^1 = Glc, R^2 = Glc^2-Glc
2: rebaudioside A R^1 = Glc, R^2 = Glc^2-Glc (3 Glc)
3: rebaudioside C R^1 = Glc, R^2 = Glc^2-Rha (^{3}Glc)
4: dulcoside A R^1 = Glc, R^2 = Glc^2-Rha
5: rubusoside R^1 = Glc, R^2 = Glc
6: steviol R^1 = H, R^2 = H



7: isosteviol



8: glycyrrhizin R = $GlcA^2$-$GlcA^1$



9: curcumin

Figure 1. Structures of compounds **1**–**9**.

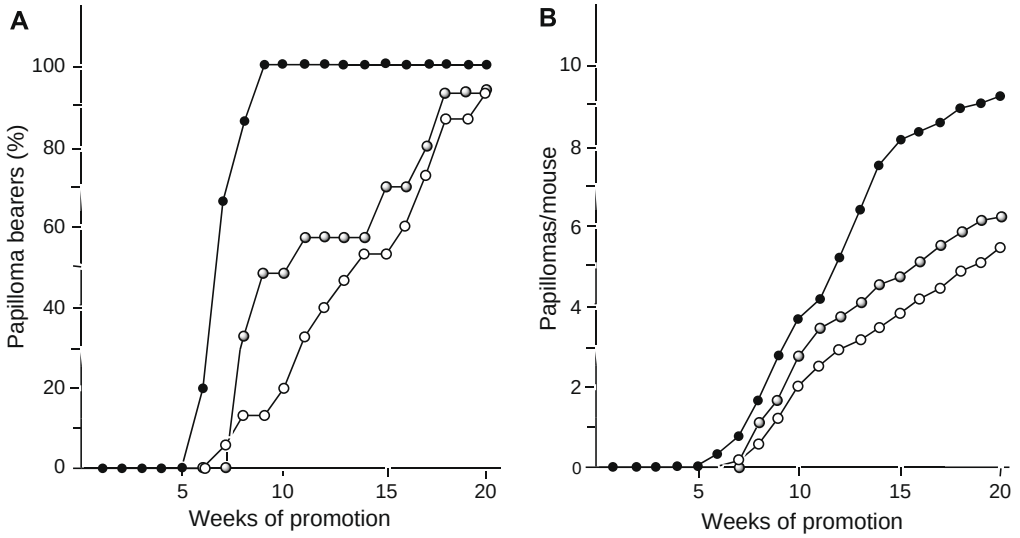


Figure 2. Inhibitory effects of stevioside (**1**) and glycyrrhizin on mouse skin carcinogenesis induced by DMBA and TPA. All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. **A**: percentage of mice bearing papillomas; **B**: average number of papillomas per mouse; group Ia: ● positive control, TPA alone; TPA + 85 nmol of stevioside; group V: ◍ TPA + 85 nmol of glycyrrhizin. At 20 weeks of promotion, group II and group V were significantly different from group Ia ($p < 0.05$, using Student's *t*-test) on papillomas per mouse.

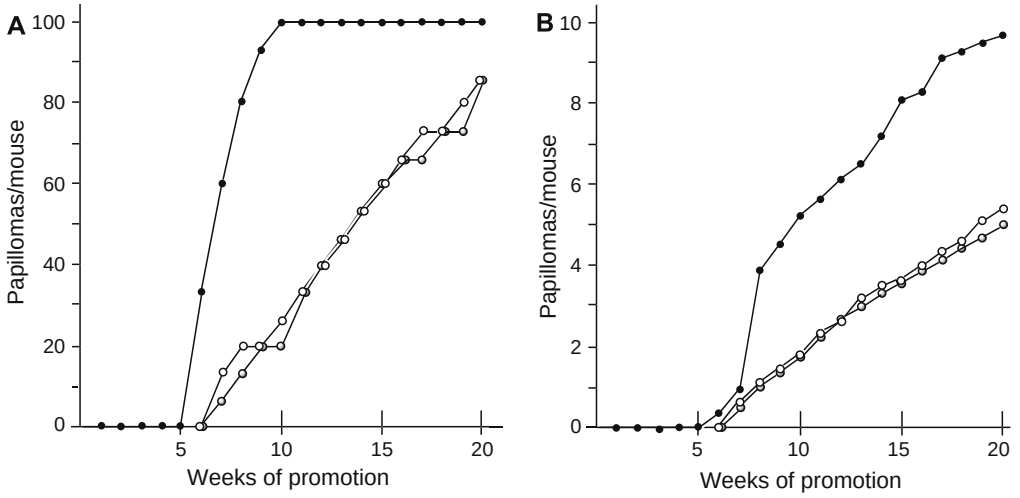


Figure 3. Inhibitory effects of steviol (**6**) and isosteviol (**7**) on mouse skin carcinogenesis induced by DMBA and TPA. All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. **A**: percentage of mice bearing papillomas; **B**: average number of papillomas per mouse; group Ib: ● positive control, group III: ○ TPA + 85 nmol of steviol; group IV: ◍ TPA + 85 nmol of isosteviol. At 20 weeks of promotion, group III was significantly different from group Ib ($p < 0.05$, using Student's *t*-test) on papillomas per mouse.

As shown in Figure 2A, in group Ia (positive control, using ICR mice), which received treatment with DMBA and TPA, the first tumor appeared after 6 weeks of promotion, and 100% of the mice bore papillomas after 9 weeks of promotion. Further, averages of 3.7, 8.1 and 9.2 papillomas were formed per mouse at 10, 15 and 20 weeks of promotion, respectively, as shown in Figure 2B. On the other hand, when **1** was applied before TPA treatment (group II), it delayed and reduced the formation of papillomas as follows: the first tumor appeared after 7 weeks of promotion and the incidence of papilloma-bearing mice was reduced to about 20%, 53% and 93% at 9, 15 and 20 weeks of promotion, respectively (Fig. 2A). Also, averages of only 2.0, 3.8 and 5.4 papillomas per mouse were recognized at 10, 15 and 20 weeks of promotion, respectively (Fig. 2B). [Previously, Yasukawa et al. described the effects of stevioside mixture on the promotion of skin papillomas by TPA in DMBA-initiated ICR mice under different experimental conditions (higher dose of test sample and lower doses of DMBA and TPA were applied).[19]]

Group III mice, which were treated with DMBA, **8** and TPA, also showed the first tumor after 7 weeks of promotion, and about 50%, 60% and 94% of the mice bore papillomas at 10, 15 and 20 weeks of promotion, respectively (Fig. 2A). Further, averages of 2.8, 4.6 and 6.2 papillomas were formed per mouse at 10, 15 and 20 weeks of promotion, respectively, as shown in Figure 2B. Therefore, **1** and **8** caused 41.3% and 35.0% reduction, respectively, in the average number of the tumors per mouse after 20 weeks of promotion compared with the positive control group.

In Figure 3, with group Ib (positive control, using SENCAR mice), treated with DMBA and TPA, the first tumor appeared after 6 weeks of promotion, and 100% of the mice bore papillomas after 10 weeks of promotion (Fig. 3A). Further, averages of 5.2, 8.1 and 9.7 papillomas were formed per mouse at 10, 15 and 20 weeks of promotion,

respectively, as shown in Figure 3B. On the other hand, in group II, treated with DMBA, **6** and TPA, the first tumor appeared after 7 weeks of promotion, and about 27%, 60% and 87% of the mice bore papillomas at 10, 15 and 20 weeks of promotion, respectively (Fig. 3A). Further, averages of 1.8, 3.7 and 5.4 papillomas were formed per mouse at 10, 15 and 20 weeks of promotion, respectively, as shown in Figure 3B. In group III, treated with DMBA, **7** and TPA, the first tumor appeared after 7 weeks of promotion, and about 20%, 60% and 87% of the mice bore papillomas at 10, 15 and 20 weeks of promotion, respectively (Fig. 3A). Furthermore, averages of 1.8, 3.6 and 5.0 papillomas were formed per mouse at 10, 15 and 20 weeks of promotion, respectively (Fig. 3B). Thus, in this assay, **6** and **7** caused 44.3% and 46.4% reduction, respectively, in the average number of the tumors per mouse after 20 weeks of promotion compared with the positive control group.

From the above results, **1**, **6** and **7** exhibited strong antitumor-promoting potency by delaying formation and reducing the number of papillomas per mouse. Further, their inhibitory effects were comparable to each other and greater than those of glycyrrhizin, which is a known antitumor-promoter isolated from licorice root. [18]

2.3. Two-stage carcinogenesis test on mouse skin induced by peroxynitrite and TPA

Figure 4 (for **1** and **7**) and Figure 5 (for **6**) show the time course of tumor formation after treatment with the test compounds. Specifically, the incidence (%) of papilloma-bearing mice and the average numbers of papillomas per mouse are presented in Figures 4A, 5A and Figures 4B, 5B respectively.

Group I (positive control), treated with peroxynitrite and TPA, exhibited the first tumor after 7 weeks, and the percentage of tumor bearing mice was 100% after 11 weeks of promotion (Fig. 4A). Further, averages of 4.3, 7.2 and 8.0 papillomas were formed per mouse at 11, 15 and 20 weeks of promotion, respectively, as shown in Figure 4B. On the other hand, in group II, treated with peroxynitrite and **1** applied before TPA treatment, the first tumor appeared after 8 weeks of promotion, and about 40%, 53% and 87% of the mice bore papillomas at 11, 15 and 20 weeks of after promotion, respectively (Fig. 4A). Further, averages of 2.0, 3.0 and 4.4 papillomas were formed per mouse at 11, 15 and 20 weeks of promotion, respectively (Fig. 4B). In group III, treated with peroxynitrite, **7** and TPA, the first tumor appeared after 8 weeks of promotion, and about 33%, 53% and 73% of the mice bore papillomas at 11, 15 and 20 weeks of promotion, respectively (Fig. 4A). Further, averages of 1.6, 2.7 and 4.3 papillomas were formed per mouse at 10, 15 and 20 weeks of promotion, respectively, as shown in Figure 4B.

As shown in Figure 5A, in group I (positive control), treated with peroxynitrite and TPA, the first tumor appeared after 6 weeks and the percentage of tumor bearing mice was 100% after 10 weeks of promotion. Furthermore, averages of 4.3, 7.4 and 8.1 papillomas were formed per mouse at 10, 15 and 20 weeks of promotion, respectively, as shown in Figure 5B. On the other hand, group II, treated with peroxynitrite, **6** and TPA, showed the first tumor after 8 weeks of promotion, and the incidence of the tumor bearer was approximately 27%, 67% and 93% at 10, 15 and 20 weeks of promotion, respectively (Fig. 5A). Further, regarding the average numbers of papillomas per mouse (Fig. 5B), 2.3, 3.5 and 4.5 papillomas were formed at 10, 15 and 20 weeks of promotion, respectively. The results of this assay showed that treatment with **1**, **6** and **7** delayed the formation of papillomas compared to the positive control group and reduced the number of tumors per mouse by 45.0%, 44.4% and 46.2%, respectively, after 20 weeks of promotion compared with the control group. These values were almost the same as that of curcumin (**9**),[20] namely, 45.1% reduction in the average number of the tumors per mouse after 20 weeks of promotion compared with control. Curcumin is known to have potent antioxidant activity; it intercepts and neutralizes potent chemical carcinogens, such as ROS (superoxide, peroxyl and hydroxy radicals) and NO donors.[21,22]

The results of these in vivo tests showed that, with comparable effects to curcumin, compounds **1**, **6** and **7** delayed formation and reduced the number of papillomas per mouse in antitumor-initiation tests. These three compounds also show almost the same inhibitory effects on tumor-initiation by peroxynitrite/TPA.

3. Conclusions

From the results of the two-stage carcinogenesis tests, it was concluded that stevioside (**1**), steviol (**6**) and isosteviol (**7**) strongly

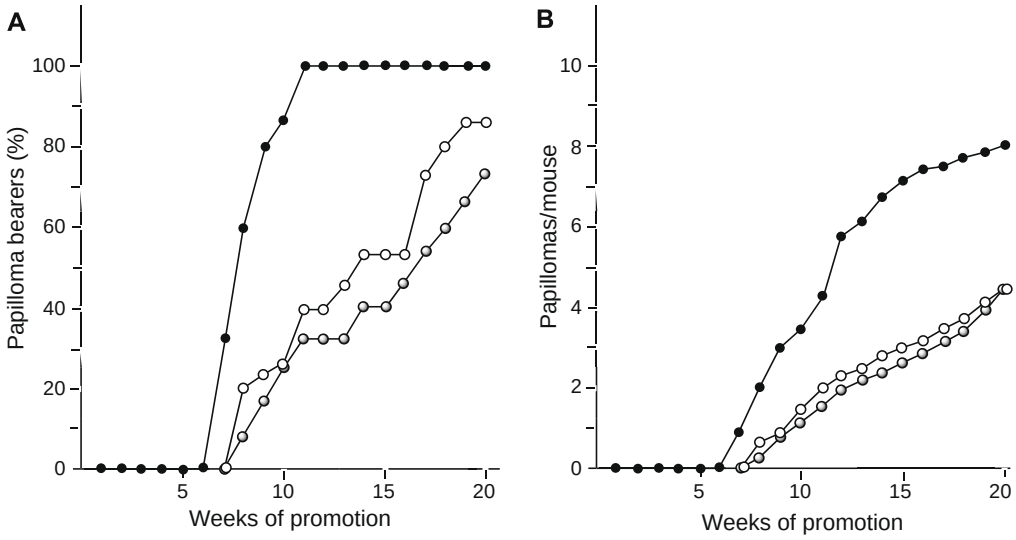


Figure 4. Inhibitory effects of stevioside (**1**) and isosteviol (**7**) on mouse skin carcinogenesis induced by peroxynitrite. All mice were initiated with peroxynitrite (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. **A**: percentage of mice bearing papillomas; **B**: average number of papillomas per mouse; group I: ● positive control, peroxynitrite nmol) + TPA alone; group II: ○ peroxynitrite (390 nmol) + 0.0025% of stevioside (two weeks) + TPA (1.7 nmol); group III: ◍ peroxynitrite (390 nmol) + 0.0025% of isosteviol (two weeks) + TPA (1.7 nmol). At 20 weeks of promotion, group II and group III were significantly different from group I ($p < 0.05$, using Student's *t*-test) on papillomas per mouse.

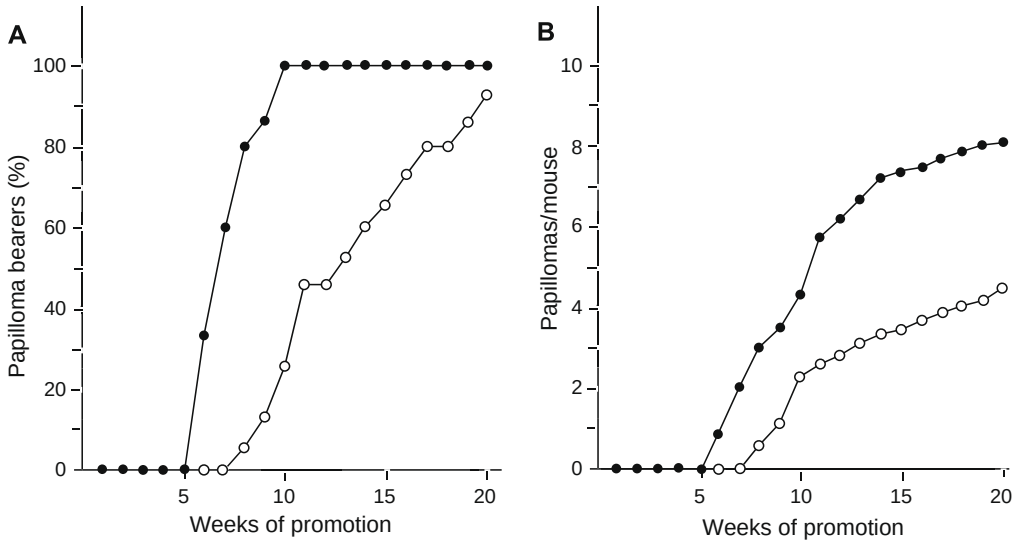


Figure 5. Inhibitory effects of steviol (**6**) on mouse skin carcinogenesis induced by peroxynitrite and TPA. All mice were initiated with peroxynitrite (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. **A**: percentage of mice bearing papillomas; **B**: average number of papillomas per mouse; group I: ●, positive control, peroxynitrite nmol) + TPA (1.7 nmol) alone; group II: ○ peroxynitrite (390 nmol) + 0.0025% of steviol (two weeks) + TPA (1.7 nmol). At 20 weeks of promotion, group II and group III were significantly different from group I ($p < 0.05$, using Student's *t*-test) on papillomas per mouse.

inhibited the promotion stage induced by DMBA/TPA and also significantly inhibited the initiation stage induced by peroxynitrite. The inhibitory effects of the three compounds in both assays were almost the same, and these results suggest that these compounds could be promising cancer preventive agents. In addition, several toxicological studies on the possible carcinogenicity or mutagenicity of **1** have been reported, in which it is neither carcinogenic nor mutagenic.[23] Therefore, **1** might be valuable not only as a natural sweetener, but also as a chemopreventive agent against chemical carcinogenesis.

4. Experimental

4.1. General

Melting points were measured on a Fisher-Johns melting point apparatus without correction. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 (300 MHz) NMR spectrometer with TMS as the internal standard. Mass spectroscopic (MS) data were obtained on TRIO 1000 mass spectrometer. IR (KBr) spectra were recorded on a Perkin–Elmer 1320 spectrometer. Analytical thin-layer chromatography (TLC) was carried out on Merck precoated aluminum silica gel sheets (Kieselgel 60 F).

4.2. Test compounds

The chemical structures are shown in Figure 1. Test samples of stevioside (**1**), rebaudioside A (**2**), rebaudioside C (**3**), dulcoside A (**4**), rubusoside (**5**) and isosteviol (**7**) were provided by Maruzen Pharmaceutical Co., Ltd Steviol (**6**) was obtained by hydrolysis of **1** using Pectinase A 'Amano' (origin: *Asperugillus oryzae* and *A. pulverulentus*). The experimental methods are as follows: stevioside (12.02 g) and Pectinase A 'Amano' (6.08 g) were divided into 36 polypropylene tubes (50 mL), and distilled water (ca. 35 mL) and 4–5 drops of toluene were added into each tube. The tubes containing the mixture were shaken for 2 weeks at 50 °C in an incubator. The hydrolytic mixture was extracted three times with diethyl ether. After drying over anhydrous magnesium sulfate, the solvent was evaporated (residue: 4.2 g). The residue was recrystallized from methanol three times, giving colorless crystals (3.2 g) mp 214–215 °C. Steviol (**6**) was identified by comparison of TLC, IR, MS, ^{1}H and ^{13}C NMR data with those of an authentic sample.

4.3. Chemicals

The cell culture reagents, *n*-butyric acid, and other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan). DMBA, TPA, and RPMI-1640 medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and peroxynitrite from Dojindo Laboratories Co. (Kumamoto, Japan). EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from Professor H. Hattori, Department of Otorhinolaryngology, Kobe University. Pectinase A 'Amano' was provided from Amano Enzyme Inc. (Nagoya, Japan).

4.4. Cells

EBV genome-carrying lymphoblastoid cells (Raji cells derived Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 under the conditions described previously.[14] Spontaneous activation of EBV-EA in our subline of Raji cells was less than 0.1%.

4.5. Animals

Specific pathogen-free (SPF) female ICR and female SENCAR mice (6 weeks old, respectively) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and maintained under SPF conditions in Animal Center of Kyoto Prefectural University of Medicine. The mice were housed five per polycarbonate cage in a temperature-controlled room at 24 ± 2 °C and given food, Oriental MF (Oriental Yeast Co., Tokyo, Japan), and water or aqueous sample solution *ad libitum* during the experiments. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kyoto Prefectural University of Medicine.

4.6. Inhibition of EBV-EA activation assay

Inhibition of EBV-EA activation was assayed using Raji cells (virus nonproducer type), an EBV genome-carrying human lymphoblastoid cell, which were cultivated in 10% fetal bovine serum

(FBS) RPMI 1640 medium. The indicator cells (Raji, 1×10^6/mL) were incubated at 37 °C for 48 h in 1 mL of medium containing *n*-butyric acid (4 mM as trigger), TPA (32 pM = 20 ng in 2 μL of DMSO as inducer), and various amounts of the test compounds dissolved in 5 μL of DMSO (ca. 0.7% DMSO). Smears were made from the cell suspension. The EBV-EA inducing cells were stained with high titer EBV-EA positive serum from NPC patients and detected by an indirect immunofluorescence technique.[7] In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. Triplicate assays were performed for each data point. The average EBV-EA induction of the test compound was expressed as IC_{50} value and as a relative ratio to the positive control experiment (100%), which was carried out with *n*-butyric acid (4 mM) plus TPA (32 pM). In the experiments, the EBV-EA induction was normally around 35%, and this value was taken as the positive control (100%). *n*-Butyric acid (4 mM) alone induced 0.1% EA-positive cells. The viability of treated Raji cells was assayed by the trypan blue staining method. The cell viability of the TPA positive control was greater than 80%. Therefore, only the compounds that induced less than 80% (% of control) of the EBV-activated cells (those with a cell viability of more than 60%) were considered able to inhibit the activation caused by promoter substances. Student's *t*-test was used for all statistical analyses.

4.7. Two-stage mouse skin carcinogenesis model induced by DMBA/TPA

Animals (6 weeks old SPF female ICR mice for **1**, 6 weeks old SPF female SENCAR mice for **6** and **7**) were divided into five experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with DMBA (100 mg, 390 nmol) in acetone (0.1 mL) as an initiation treatment. For group Ia (positive control group of the ICR mice) and group Ib (positive control group of the SENCAR mice), one week after the initiation, papilloma formation was promoted twice a week by the application of TPA (1 mg, 1.7 nmol) in acetone (0.1 mL) on the skin. For groups II, III, IV and V, test samples [**1**, **6**, **7** and glycyrrhizin (**8**) (85 nmol each)] in acetone (0.1 mL) were topically applied for 1 h before the each promotion treatment.

The incidence of papilloma bearers and numbers of papillomas per mouse were observed weekly for 20 weeks: the percentage of mice bearing papillomas and the average number of papillomas per mouse were recorded. A pathologist checked the type of tumor in these experiments by histological examination. Statistical significance was determined using Student's *t*-test.

4.8. Two-stage mouse skin carcinogenesis test induced by peroxynitrite and TPA

Animals (6 weeks old SPF female SENCAR mice) were divided into five experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with acetone (0.1 mL) and after 10 s, peroxynitrite (33.1 μg, 390 nmol in 0.1 mL of 1 mM NaOH) as an initiation treatment.

For group I (positive control group), one week after the initiation, papilloma formation was promoted by the twice weekly application of TPA (1 μg, 1.7 nmol) in acetone (0.1 mL) on the skin (no papilloma formation was seen with topical application of the acetone solvent alone). For groups II, III, IV and V, test samples [**1**, **6**, **7** and curcumin (**9**) (each 0.0025% in drinking water)] were orally administered (average 7.5 mL per mouse per day) for two weeks before the promotion treatment (from one week before initiation to one week after initiation). Subsequently, each group was promoted by the twice a week application with TPA (1 μg, 1.7 nmol) in acetone (0.1 mL). The incidence of papilloma bearers and numbers of papillomas per mouse were detected weekly for 20 weeks: the percentage of mice bearing papillomas and the average number of papillomas per mouse were recorded. Student's *t*-test was used for statistical analyses of the numbers of papillomas per mouse. The animal weights were not statistically different between any of the groups in all in vivo assays.

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