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Cancer-chemopreventive effects of natural sweeteners and related compounds*

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Abstract: To search for possible cancer-chemopreventive agents from natural resources, several natural sweeteners were screened by the in vitro assay indicated by the inhibitory effects of Epstein-Barr virus early antigen (EBV-EA) induction. Of active compounds that showed the remarkable inhibitory effects on the EBV-EA induction, stevioside, from the leaves of *Stevia rebaudiana*, and mogroside V, from the fruits of *Momordica grosvenori*, exhibited significant inhibitory effects on the two-stage mouse skin carcinogenesis in vivo induced by 7,12-dimethylbenz[a]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The inhibitory effect of stevioside is stronger than that of glycyrrhizin, which had been known as an antitumor-promoter in chemical carcinogenesis. Furthermore, stevioside also inhibited mouse skin carcinogenesis initiated by peroxynitrite. These results suggest that stevioside and mogroside V might be valuable as chemopreventive agents for chemical carcinogenesis.

INTRODUCTION

Although many kinds of antitumor agents and their related compounds have been isolated from natural resources, and the medicinal sciences have made rapid progress in the treatment of carcinogenesis, cancer currently remains a tragic disease and is one of the major causes of death worldwide. Furthermore, perfectly effective antitumor agents free from side actions have not been found yet, and these side actions of antitumor agents are a serious problem in the treatment of cancer. Therefore, the advancement of chemoprevention is important, as well as the development of cancer treatment. For chemoprevention, the inhibition of the tumor promotion stage in the multistage of chemical carcinogenesis has been regarded as the one of the most promising methods. On the other hand, it has been ascertained that the overproduction of nitric oxide or NO radicals induced mutagenesis on genes and strongly initiated the multistage carcinogenesis [1].

From these points of view, we have been studying cancer-chemopreventive agents from natural compounds, indicating their antitumor-promoting and antitumor-initiating effects [2–5]. As a continuation of our biological studies on the potential chemopreventive agents of natural products, many diterpenoid glycosides [6] and triterpenoid glycosides [7–9] were examined using both in vitro primary screening test [using Epstein-Barr virus early antigen (EBV-EA) induction by a tumor promoter, TPA] and in vivo two-stage carcinogenesis test (using DMBA or peroxynitrite as an initiator and TPA as a promoter). The cancer-chemopreventive effects of glycyrrhizin and monoglucuronide of glycyrrhetic acid on mouse skin and pulmonary and hepatic tumors had been reported [10]. In this paper, the inhibitory effects of a sweet diterpenoid glycoside, stevioside from the leaves of *Stevia rebaudiana* [11], and a sweet triterpenoid glycoside, mogroside V from the fruits of *Momordica grosvenori* [12], on

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mouse skin carcinogenesis and the possibility of these natural sweeteners for cancer-chemopreventive agents in chemical carcinogenesis are reported.

EXPERIMENTAL

In vitro assay method on the inhibition of EBV-EA induction

The inhibition of EBV-EA induction was assayed using Raji cells, EBV genome-carrying human lymphoblastoid cell, which were cultivated in 10 % fetal bovine serum 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), TPA (32 nM), and various amounts of the test compounds dissolved in DMSO (5 μL). 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. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. The EBV-EA inhibitory activity of the test compound was expressed by comparison with that of the positive control experiment (100 %). In the experiments, the EBV-EA induction was ordinarily around 35 %, and this value was taken as the positive control (100 %). The viability of treated Raji cells was assayed by the Trypan blue staining method.

In vivo assay method on two-stage mouse skin carcinogenesis induced by DMBA and TPA

The animals (specific pathogen-free female ICR, 6 weeks old) were divided into 5 experimental groups, 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were topically treated with DMBA (100 μg, 390 nmol) in acetone (0.1 ml) as an initiation treatment. For group I (positive control group), one week after initiation with DMBA, mice were promoted by the application with TPA (1 μg, 1.7 nmol) in acetone (0.1 ml) twice a week. Group II received a topical application of tested compound (85 nmol) 1 h before each promotion treatment. The incidence of papillomas was observed weekly for 20 weeks: The percentage of mice bearing papillomas and the average number of papillomas per mouse were recorded. The type of tumors in this experiment were checked by the pathologist with histological examination. Statistical significance was determined using Studen's *t*-test.

In vivo assay method on two-stage mouse skin carcinogenesis induced by peroxynitrite and TPA

The animals (female SENCAR, 6 weeks old) were divided into 2 experimental groups, 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were topically treated with peroxynitrite (33.1 μg, 390 nmol, 1 mM NaOH) in acetone (0.1 ml) as an initiation treatment. For group I (positive control group), one week after initiation with peroxynitrite, mice were promoted by the application with TPA (1 mg, 1.7 nmol) in acetone (0.1 ml) twice a week. For group II, stevioside (0.0025 %, 2.5 mg/100 ml) in drinking water was given, from one week before to one week after the initiation treatment with peroxynitrite, and then promoted by the application with TPA (1 μg, 1.7 nmol) in acetone (0.1 ml) twice a week. The incidence of papillomas was observed weekly for 20 weeks: The percentage of mice bearing papillomas and the average number of papillomas per mouse were recorded.

RESULTS AND DISCUSSION

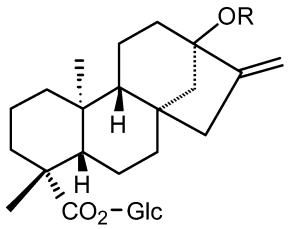
Sweet diterpenoid, stevioside

As shown in Table 1, the primary screening test in vitro of 5 sweet diterpenoids obtained from the leaves of *Stevia rebaudiana* was carried out using a short-term synergistic assay on EBV-EA induction with

Table 1 Percentages of Epstein-Barr virus early antigen induction in presence of diterpenoids with respect to positive control.

Samples	Concentration[a]			
	1000	500	100	10
Stevioside	3.2[b] (60)[c]	36.9 (>80)	67.4 (>80)	89.3 (>80)
Rebaudioside A	14.8 (60)	47.3 (>80)	82.5 (>80)	100 (>80)
Rebaudioside C	15.4 (60)	46.9 (>80)	83.7 (>80)	100 (>80)
Dulcoside A	6.7 (60)	40.9 (>80)	73.0 (>80)	92.5 (>80)
Rubusoside	11.4 (60)	42.6 (>80)	75.7 (>80)	93.2 (>80)

[a]Mol ratio/TPA (20 ng = 32 pmol/ml).
[b]Values represent relative percentages to the positive control values (100 %).
[c]Values in parentheses are viability percentages of Raji cells.



stevioside R = –Glc(2)–Glc

rebaudioside A R = –Glc(2)–Glc, Glc(3)–Glc

rebaudioside C R = –Glc(2)–Rha, Glc(3)–Glc

dulcoside A R = –Glc(2)–Rha

rubusoside R = –Glc

TPA. Of these diterpenes, stevioside exhibited the strongest inhibitory effects on EBV-EA induction (more than 95, 63, and 32 % inhibition at 1×10^3, 5×10^2, and 1×10^2 mol ratio/TPA, respectively), and preserved a high viability of Raji cells. In our experiment, the inhibitory effect of stevioside was stronger than that of glycyrrhizin, which has been known as an antitumor-promoting reagent [13]. Based on our experiments in which many natural products which strongly inhibited, EBV-EA induced by TPA exhibited a remarkable antitumor-promoting effect on the two-stage carcinogenesis [2–9], the inhibitory effect of stevioside was expected and was investigated by a two-stage carcinogenesis test of mouse skin tumors induced by DMBA as an initiator and TPA as a promoter.

As shown in Fig. 1, in the positive control group, which received treatment with DMBA and TPA, there was 100 % incidence of papillomas within 9 weeks of promotion (Fig. 1A: % of papilloma bearers). In the group treated with DMBA, TPA, and glycyrrhizin, about 50, 70, and 94 % of mice bore papillomas at 10, 15 and 20 weeks of promotion, respectively. The animals treated with DMBA, TPA, and stevioside for 10 weeks showed papilloma formation of less than 20 %, by 15 weeks showed 53 %, and by 20 weeks showed 94 %. Furthermore, these tumor-inhibitory effects were also seen as a reduction in the multiplicity of papillomas (Fig. 1B: the number of papillomas per mouse) over a 15-week period. In the positive control group, 8.1 and 9.2 papillomas were formed per mouse after 15 and 20 weeks of promotion, respectively. In the groups treated with DMBA, TPA, and stevioside, fewer than 3.8 and 5.4 papillomas were formed per mouse after the same respective periods.

These results showed that when stevioside was applied before each TPA treatment, the formation of papillomas on mouse skin was significantly delayed and the number of papillomas was remarkably reduced. As shown in Figs. 1A and B, the inhibitory effects of stevioside on two-stage carcinogenesis of mouse skin tumor was more potent than that of glycyrrhizin.

Furthermore, on the basis of experimental results that stevioside exhibited the inhibitory effect on the point-mutation of H-ras gene from mouse skin induced by the treatment with peroxynitrite ($ONOO^-$), the inhibition of stevioside on the initiation stage of mouse skin carcinogenesis was

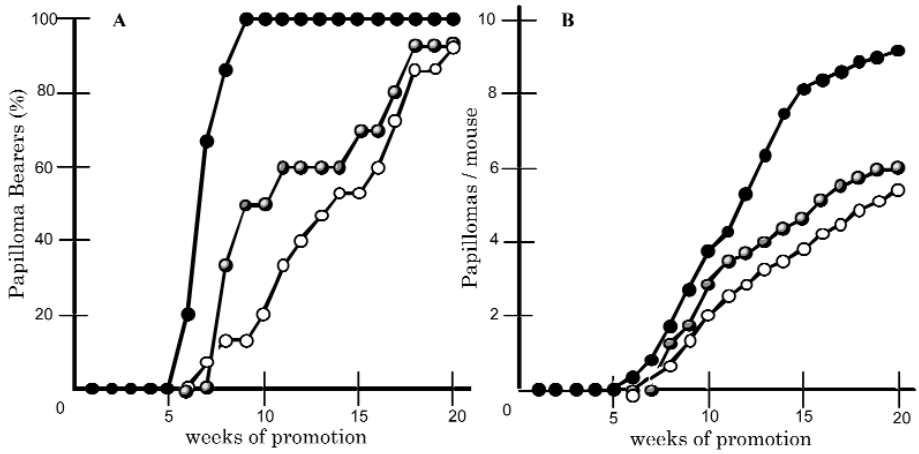


Fig. 1 Inhibitory effects of glycyrrhizin and stevioside on mouse skin carcinogenesis induced by DMBA and TPA. All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas; B: average number of papillomas per mouse; ●, positive control, TPA alone; ●, TPA + 85 nmol of glycyrrhizin; ○, TPA + 85 nmol of stevioside. At 10 and 15 weeks of promotion, the group treated with stevioside was significantly different from the positive control group ($p < 0.01$, using Studen's *t*-test) in terms of papilloma bearers (%), and at 15 and 20 weeks of promotion, the group treated with stevioside was different from control group ($p < 0.01$, using Studen's *t*-test) in terms of papillomas per mouse ($n = 15$, control group: 8.1 ± 0.6 and 9.2 ± 0.7 and the group treated with stevioside: 3.8 ± 0.2 and 5.4 ± 0.4).

expected. The inhibitory effect of stevioside on the two-stage carcinogenesis of mouse skin using peroxynitrite as an initiator was investigated.

As shown in Fig. 2, stevioside, which was applied by oral administration for only 2 weeks, exhibited a significant inhibitory effect on the tumor-initiation induced by peroxynitrite. In the positive control group, which received treatment with peroxynitrite and TPA showed 87 and 100 % incidence of papillomas in less than 10 or 11 weeks (Fig. 2A: % of papilloma bearers). The test animals, which had ingested 0.0025 % stevioside for 2 weeks (from 1 week before initiation to 1 week after initiation with peroxynitrite), took 10 weeks to show only 30 %, 15 weeks to show 53 % and even 20 weeks to show 86 % papilloma formation. As shown in the multiplicity of papillomas per mouse (Fig. 2B), stevioside also apparently reduced the number of papillomas over a 10-week period. In the positive control group, 3.5, 7.2, and 8.0 papillomas were formed per mouse after 10, 15, and 20 weeks of promotion, respectively. On the other hand, in the group treated with 0.0025 % stevioside, only 1.5, 3.0, and 4.4 were formed per mouse after 10, 15, and 20 weeks of promotion, respectively.

From these results of two-stage carcinogenesis test, it was concluded that sweet diterpenoid, stevioside, inhibited the promotion stage on the two-stage mouse skin carcinogenesis induced by TPA and also inhibited the initiation stage induced by peroxynitrite. On the other hand, several toxicological studies on the carcinogenicity or mutagenic activity have also been reported, in which stevioside is not carcinogenic and not mutagenic [14–16]. Therefore, stevioside might be a valuable natural sweetener as a chemopreventive agent against chemical carcinogenesis.

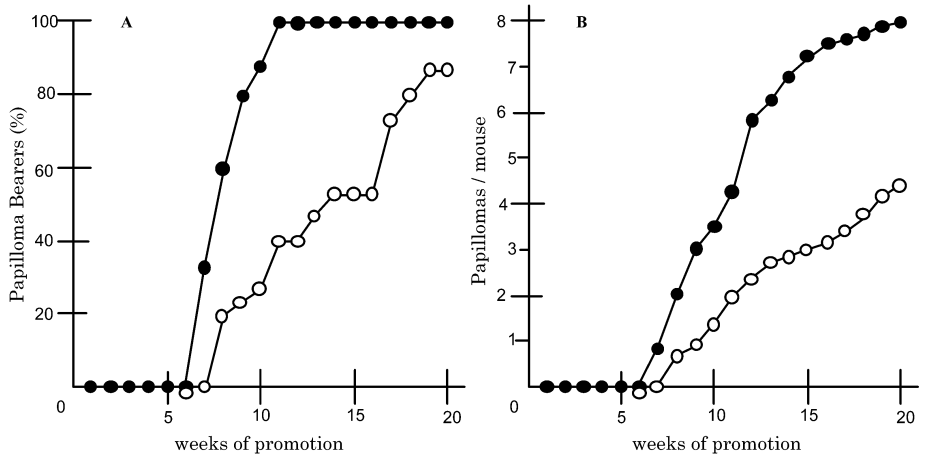


Fig. 2 Inhibitory effects of stevioside on mouse skin carcinogenesis induced by peroxynitrite and TPA. All mice were initiated with peroxynitrite (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas; B: average number of papillomas per mouse; ●, positive control, peroxynitrite (390 nmol) + TPA (1.7 nmol) alone; ○, peroxynitrite (390 nmol) + 0.0025 % stevioside (2 weeks) + TPA (1.7 nmol). At 10 and 15 weeks of promotion, the group treated with stevioside was significantly different from the positive control group ($p < 0.01$, using Studen's *t*-test) in terms of papilloma bearers (%), and at 10, 15, and 20 weeks of promotion, the group treated with stevioside was different from control group ($p < 0.01$, using Studen's *t*-test) in terms of papillomas per mouse ($n = 15$, control group: 3.5 ± 0.2, 7.2 ± 0.5, and 8.0 ± 0.6 and the group treated with stevioside: 1.5 ± 0.1, 3.0 ± 0.1, and 4.4 ± 0.2).

Sweet cucurbitane glycoside, mogroside V

Many cucurbitane glycosides had been isolated from several cucurbitaceous plants [17–19], and their taste had also been examined and reported [20]. Of these cucurbitane glycosides, carnosifloside V, VI obtained from *Hemsleya carnosiflora*, scandenoside R6 from *H. panacis-scandens*, mogroside IV, V, and siamenoside I from *Momordica grosvenori* have sweet taste. The primary screening test in vitro of these sweet cucurbitane triterprnoids was carried out using a short-term synergistic assay on EBV-EA induction with TPA, and the results are shown in Table 2.

Of these glycosides, scandenoside R6 exhibited the strongest inhibitory effect on EBV-EA induction, and the inhibitory effect of mogroside V is almost the same as that of glycyrrhizin, which has been known as an antitumor-promoter. The inhibitory effects of scandenoside R6 and mogroside V on two-stage mouse skin carcinogenesis induced by DMBA and TPA were examined, and their effects are shown in Fig. 3.

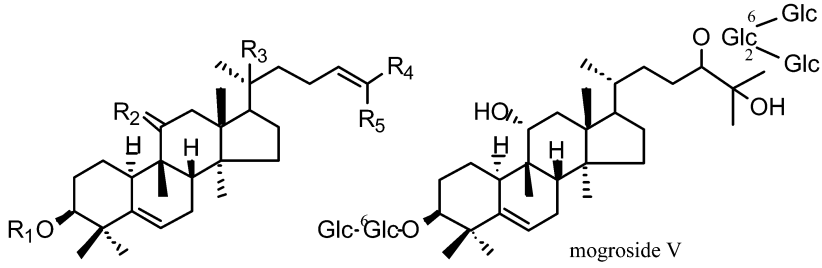
In the group treated with DMBA, TPA, and mogroside V, only about 40, 60, and 87 % of the mice bore papillomas at 10, 15, and 20 weeks of promotion, respectively (Fig. 3A). In the positive control group, 100 % of the mice bore papillomas within 9 weeks of promotion, therefore, the treatment of mogroside V apparently delayed the papilloma formation on two-stage mouse skin carcinogenesis promoted by TPA. Further, mogroside V caused 50 % reduction in the number of papillomas per mouse over a 15-week period (Fig. 3B), indicating its inhibitory potential toward DMBA and TPA-induced two-stage skin carcinogenesis. This inhibitory effect of mogroside V on two-stage carcinogenesis of mouse skin tumor was more potent than that of glycyrrhizin. On the other hand, against our expectation, the inhibitory effects of scandenoside R6 in vivo are less than mogroside V.

The fruit of *Momordica grosvenori* has been cultivated in restricted areas of the southern part of China, Guangxi province, and only heated and dried fruits or extract were traded to other areas and

Table 2 Percentages of Epstein-Barr virus early antigen induction in presence of cucurbitane glycosides with respect to positive control.

Samples	Concentration[a]			
	1000	500	100	10
Carnosifloside I	28.5[b] (>80)[c]	63.8 (>80)	82.5 (>80)	100 (>80)
Carnosifloside V[d]	18.3 (70)	35.4 (>80)	72.5 (>80)	95.9 (>80)
Carnosifloside VI[d]	20.7 (>80)	45.8 (>80)	79.9 (>80)	100 (>80)
Scandenoside R2	33.5 (>80)	59.8 (>80)	90.6 (>80)	100 (>80)
Scandenoside R4	31.5 (70)	55.3 (>80)	89.6 (>80)	100 (>80)
Scandenoside R6[d]	0.0 (>80)	25.3 (>80)	70.3 (>80)	94.6 (>80)
Mogroside V[d]	20.8 (>80)	51.5 (>80)	84.6 (>80)	100 (>80)
Glycyrrhizin[d]	26.4 (>80)	63.5 (>80)	82.3 (>80)	100 (>80)

[a]Mol ratio/TPA (20 ng = 32 pmol/ml).
[b]Values represent relative percentages to the positive control values (100 %).
[c]Values in parentheses are viability percentages of Raji cells.
[d]Sweet glycosides.



	R_1	R_2	R_3	R_4	R_5
carnosifloside I	−H	=O	−H	$-CH_2O-Glc^{6}-Glc$	$-CH_3$
carnosifloside V	−Glc	α-OH	−H	$-CH_2O-Glc^{2}-Glc$	$-CH_3$
carnosifloside VI	−Glc	α-OH	−H	$-CH_2O-Glc^{6}-Glc$	$-CH_3$
scandenoside R2	−Glc	=O	-OH	$-CH_3$	$-CH_2OH$
scandenoside R4	−Glc	=O	-OH	$-CH_3$	$-CH_2O$ Glc
scandenoside R6	−Glc	α-OH	−H	$-CH_3$	$-CH_2O\ Glc^{2}-Glc$

countries such as Southeast Asia, Japan, and the United States. The extract of this fruit has been used only for the treatment of pharyngitis or pharyngeus pain, and antitussive medicine in Japan, but it has not been used for a natural sweetener substitute for sucrose owing to its higher price and lower distribution than stevioside. However, mogroside V, which consists of the major constituent in the extract, is more than 400 times as sweet as sucrose [12], and its quality of taste is better than glycyrrhizin. This fruit and mogroside V will be valuable as an advanced food ingredient and a natural sweetener substitute for sucrose in the near future, and might be valuable as a source of the chemopreventive agents in chemical carcinogenesis.

The effects of mogroside V on the initiation stage of two-stage carcinogenesis induced by NO radicals or peroxynitrite are being studied, and the investigations in details of the inhibitory mechanism of stevioside and mogroside V on chemical carcinogenesis are also now underway.

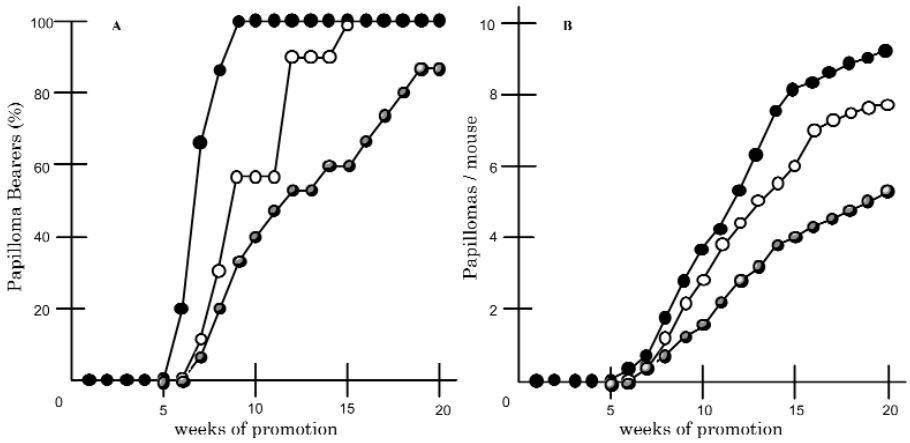


Fig. 3 Inhibitory effects of scandenoside R6 and mogroside V on mouse skin carcinogenesis induced by DMBA and TPA. All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas, B: average number of papillomas per mouse. ●, positive control, TPA alone; ○, TPA + 85 nmol of scandenoside R6; ●, TPA + 85 nmol of mogroside V. At 10 and 15 weeks of promotion, the group treated with mogroside V was significantly different from the positive control group ($p < 0.01$, using Studen's *t*-test) in terms of papilloma bearers (%), and at 15 and 20 weeks of promotion, the group treated with mogroside V was different from control group ($p < 0.01$, using Studen's *t*-test) in terms of papillomas per mouse ($n = 15$, control group: 8.1 ± 0.6 and 9.2 ± 0.7 and the group treated with mogroside V: 4.0 ± 0.2 and 5.3 ± 0.4).

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