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Cancer Letters 198 (2003) 37–42
CANCER Letters
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Anticarcinogenic activity of natural sweeteners, cucurbitane glycosides, from *Momordica grosvenori*

Midori Takasaki[a,*], Takao Konoshima[a], Yuji Murata[b], Masaki Sugiura[b], Hoyoku Nishino[c], Harukuni Tokuda[c], Kazuhiro Matsumoto[d], Ryoji Kasai[d], Kazuo Yamasaki[d]

[a]*Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan*
[b]*Biochemical Laboratory, Saraya Co., LTD, Higashisumiyoshi-ku, Osaka 546-0013, Japan*
[c]*Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-0841, Japan*
[d]*Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima 734-8551, Japan*

Received 20 February 2002; received in revised form 2 April 2003; accepted 7 April 2003

Abstract

To search for cancer chemopreventive agents from natural resources, many phytochemicals and food additives have been screened. Consequently, two natural sweeteners, mogroside V and 11-oxo-mogroside V isolated from the fruits of *Momordica grosvenori*, exhibited strong inhibitory effect on the primary screening test indicated by the induction of Epstein-Barr virus early antigen (EBV-EA) by a tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). These sweet glycosides, having cucurbitane triterpenoid aglycon, exhibited the significant inhibitory effects on the two-stage carcinogenesis test of mouse skin tumors induced by peroxynitrite ($ONOO^-$) as an initiator and TPA as a promoter. Further, 11-oxo-mogroside V also exhibited the remarkable inhibitory effect on two-stage carcinogenesis test of mouse skin tumor induced by 7,12-dimethylbenz[*a*]-anthracene (DMBA) as an initiator and TPA as a promoter.

Keywords: Cancer chemoprevention; Mogroside V; 11-oxo-mogroside V; *Momordica grosvenori*; Peroxynitrite; Anti-tumor-initiator

1. Introduction

Several natural sweeteners, such as stevioside and glycyrrhizin, have been used for safe and low-calorie sweetener (sugar substitute). In the course of our continuing search for novel cancer chemopreventive agents from natural sources [1–3], other than dietary effects, the potential anti-carcinogenic effects of these natural sweeteners were examined. Of these natural sweeteners, stevioside has exhibited the remarkable inhibitory effects on two-stage carcinogenesis induced by 7,12-dimethylbenz[*a*] anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [4]. Further, many kinds of cucurbitane glycosides which have sweet taste had been isolated from *Cucurbitaceous* plants [5,6], and mogroside V and its

* Corresponding author. Tel.: +81-75-595-4645; fax: +81-75-595-4754.
E-mail address: midori@mb.kyoto-phu.ac.jp (M. Takasaki).

0304-3835/03/$ - see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/S0304-3835(03)00285-4

derivative were isolated from the fruits of *Momordica grosvenori* (Cucurbitaceae) [7]. The fruits of *M. grosvenori* has been cultivated in restricted area of the southern part of China, and used for the treatment of pharyngitis or pharyngeus pain, and antitussive medicine in China and Japan as a folk medicine. The sweet elements of this plants have been also assayed for oxidative modification of low-density-lipoprotein, and it was reported that, of these compounds, 11-oxo-mogroside V exhibited the strongest effect [8]. To search for possible cancer chemopreventive agents from natural sources, the inhibitory effect of sweet glycosides obtained from *M. grosvenori* were screened by synergestic assay using Epstein-Barr virus as indicator cells. Further, the anti-carcinogenic activity of 11-oxo- mogroside V was examined by two-stage carcinogenesis test using DMBA as an initiator and TPA as a promoter. On the other hand, it has been ascertained that the over-produced nitric oxide (NO) is changed to peroxy-nitrite and induce any damage on gene, cell and tissue level to mutagenesis and carcinogenesis. On the basis of the experimental proof that NO and peroxynitrite strongly initiated the multi-stage carcinogenesis [9,10], the inhibitory effects of sweet cucurbitane glycosides, mogroside V and 11-oxo-mogroside V, on two-stage carcinogenesis initiated by peroxynitrite were also examined (Fig. 1).

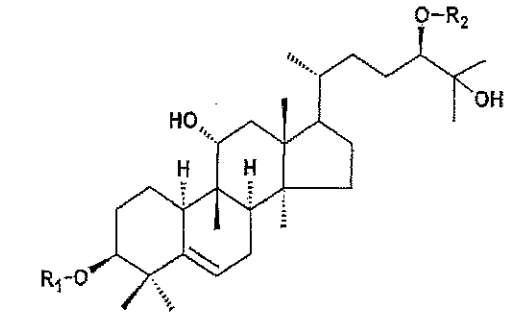
2. Material and method

2.1. Chemicals

TPA and DMBA were purchased from Sigma Chemical Co. (St Louis, USA), and Wako Pure Chemical Industries (Osaka, Japan), respectively. Peroxynitrite was purchased from Dojindo Laboratories (Kumamoto, Japan). EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medical University, Japan. Mogroside V, 11-oxo-mogroside V, mogroside IV and siamenoside I were isolated from the fruits of *M. grosvenori* according to the reported method and identified with authentic samples [7].

2.2. Cells

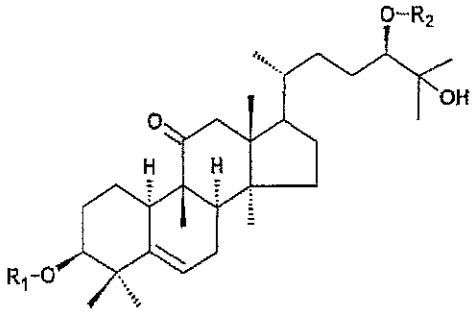
The EBV genome-carrying lymphoblastoid cells (Raji cells derived fron Birkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd, Hamamatsu, Japan) under previous described conditions [1–4]. Spontaneous activation of EBV-EA in our subline Raji cells was less than 0.1%.



mogroside IV : R_1 = —Glc^{6}—Glc R_1 = —Glc^{2}—Glc

mogroside V : R_1 = —Glc^{6}—Glc R_1 = —Glc^{2}(6|Glc)—Glc

siamenoside I : R_1 = —Glc R_1 = —Glc^{2}(6|Glc)—Glc



11-oxo-mogroside V :

R_1 = —Glc^{6}—Glc R_1 = —Glc^{2}(6|Glc)—Glc

Fig. 1. Structures of sweet glycosides isolated from the fruits of *M. grosvenori*.

2.3. Animals

Specific pathogen-free female ICR (6 weeks old) and female SENCAR (6 weeks old) mice were obtained from Japan SLC Inc. (Hamamatsu, Japan). These animals were housed, 5 per polycarbonate cage, in a temperature-controlled room at 24 ± 2 °C and given food and water ad libitum.

2.4. Inhibition of EBV-EA activation assay

The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type) as described previously [1–4]. 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 mmol), TPA (32 pmol = 20 ng) in dimethyl sulfoxide (DMSO, 2 μl), as inducer and various amounts of test compounds in 5 μl DMSO. Smears were made from the cell suspension, and the activated cells which were stained by EBV-EA positive serum from NPC patients were detected by an indirect immunofluorescence technique. For each compounds, assay were performed in triplicate. The average EBV-EA induction of the test compounds was expressed as a relative ratio to the control experiment (100%) which was carried out only with *n*-butyric acid and TPA. The viability of treated Raji cells was assayed by the Trypan-Blue staining method.

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

The animals (specific pathogen-free female ICR, 6 weeks old) were divided into two groups, 15 mice each. The back of each mouse was shaved with surgical clipper, and the mice were topically treated with DMBA (100 μg, 390 nmol) in acetone (0.1 ml) as an initiation treatment. For groups I (control group) and II, 1 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. For group II, mice were treated with 11-oxo-mogroside V (85 nmol) by a topical application, 1 h before each promotion treatment with TPA. The incidence of papillomas was observed weekly for 20 weeks; the percentages of mice bearing papillomas (Fig. 2A) and the average number of papillomas per mouse (Fig. 2B) were recorded. The type of tumors in this experiment were checked by the pathologist with the histological

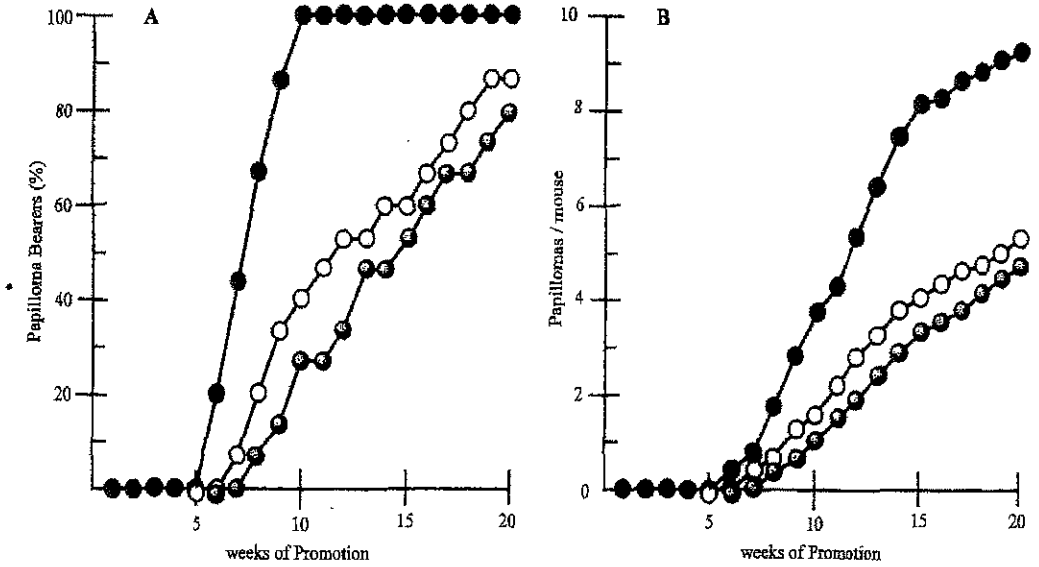


Fig. 2. Inhibitory effects of mogroside V and 11-oxo-mogroside V on mouse skin carcinogenesis induced by DMBA and TPA. (●) (control group I), DMBA (390 nmol) + TPA (1.7 nmol); (○) (reported data [4]), DMBA (390 nmol) + mogroside V (85 nmol) + TPA (1.7 nmol); (❍) (Group II), DMBA (390 nmol) + 11-oxo-mogroside V (85 nmol) + TPA (1.7 nmol). At both 15 and 20 weeks of promotion, mogroside V and 11-oxo-mogroside V treated groups were different from the control group ($p < 0.01$) in terms of papillomas per mouse ($n = 15$ and at 15 weeks of promotion, control group: 8.1 ± 0.4, mogroside V treated group 4.0 ± 0.2, and 11-oxo-mogroside V treated group 3.3 ± 0.1, and at 20 weeks of promotion, control group 9.2 ± 0.5, mogroside V treated group 5.3 ± 0.3, and 11-oxo- mogroside V treated group 4.7 ± 0.4).

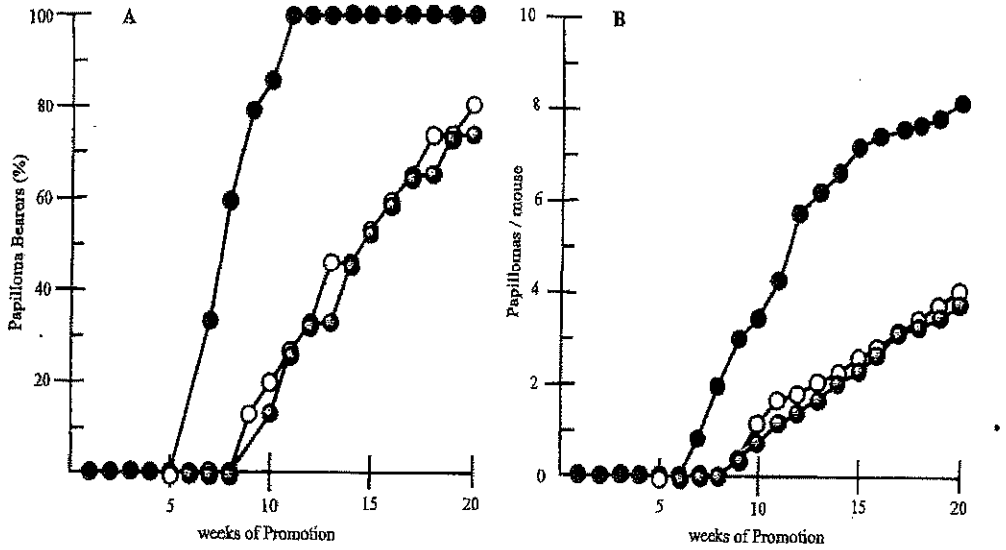


Fig. 3. Inhibitory effects of mogroside V and 11-oxo-mogroside V on mouse skin carcinogenesis induced by peroxynitrite and TPA. (●) (control group I), peroxynitrite (390 nmol) + TPA (1.7 nmol); (○) (group II), peroxynitrite (390 nmol) + 0.0025% mogroside V (in drinking water, 2 weeks) + TPA (1.7 nmol); (❍) (group III), peroxynitrite (390 nmol) + 0.0025% 11-oxo- mogroside V (in drinking water, 2 weeks) + TPA (1.7 nmol). At both 15 and 20 weeks of promotion, mogroside V and 11-oxo-mogroside V treated groups were different from the control group ($p < 0.01$) in terms of papillomas per mouse ($n = 15$ and at 15 weeks of promotion, control group: 7.2 ± 0.4, mogroside V treated group 2.6 ± 0.1, and 11-oxo-mogroside V treated group 2.5 ± 0.1, and at 20 weeks of promotion, control group 8.3 ± 0.5, mogroside V treated group 4.1 ± 0.3, and 11-oxo-mogroside V treated group 3.8 ± 0.3).

examination, and the malignant tumors were not observed at 20 weeks of promotion in our experimental system (usually, the malignant tumor could be observed at 30 weeks of promotion). The tumor incidence was statistically analyzed by Student's *t*-test in treated mice and controls.

2.6. *Two-stage mouse skin carcinogenesis test initiated by peroxynitrite*

The animals (female SENCAR, 6 weeks old) were divided into three groups, 15 mice each. The back of each mouse was shaved with surgical clipper, 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 groups I (control group) II and III, 1 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 groups II and III, mogroside V and 11-oxo-mogroside V (0.0025%, 2.5 mg/100 ml) in drinking water was given orally, from 1 week before to 1 week after the initiation treatment with peroxynitrite, respectively. The incidence of papillomas was observed weekly for 20 weeks; the percentages of mice bearing papillomas (Fig. 3A) and the average number of papillomas per mouse (Fig. 3B) were recorded. The type of tumors in our experiment is also checked by the pathologist with the histological examination, and the malignant tumors were not observed at 20 weeks of promotion in our experimental system. The tumor incidence was statistically analyzed by Student's *t*-test in treated mice and controls.

3. Results and discussion

As shown in Table 1, among four sweet glycosides from *M. grosvenori*, 11-oxo-mogroside V exhibited the strongest inhibitory effect on EBV-EA induction (91.2, 50.9 and 21.3% inhibition at 1000, 500 and 100 mol ratio/TPA concentration, respectively) and mogroside V exhibited remarkable inhibitory effect (89.9, 50.0 and 20.6% inhibition at 1000, 500 and 100 mol ratio/TPA concentration, respectively). In our past work, inhibitory effects on EBV-EA induction by TPA have correlated well with anti-tumor-promoting activity in vivo [11–15]. Further, these two glycosides are major constituents

Table 1
Percentages of EBV-EA induction in the presence of sweet glycosides from *M. grosvenori* with respect to the control (100%)

Sample	Concentration (mol ratio/TPA)[a]			
	1000	500	100	10
Mogroside IV	11.5 ± 1.2[b](60)[c]	52.0 ± 1.7 (>80)	82.1 ± 1.4 (>80)	100 ± 0 (>80)
Mogroside V	10.1 ± 0.9 (60)	50.0 ± 1.4 (>80)	79.4 ± 1.6 (>80)	98.8 ± 0.8 (>80)
Siamenoside I	13.7 ± 1.2 (60)	54.7 ± 2.0 (>80)	86.7 ± 1.1 (>80)	100 ± 0 (>80)
11-oxo-mogroside V	8.8 ± 0.8 (60)	49.1 ± 1.8 (>80)	78.7 ± 1.7 (>80)	98.5 ± 0.7 (>80)

[a] Mole ratio/TPA (32 pmol = 20 ng/ml), 1000 mol ratio = 32 nmol, 500 mol ratio = 16 nmol, 100 mol ratio = 3.2 nmol and 10 mol ratio = 0.32 nmol.
[b] Values represent percentages of EBV-EA induction to the positive control value (100%) ($n = 3$ and ± SD).
[c] Values in parentheses represent viability percentages of Raji cells.

(mogroside V: 0.45% and 11-oxo-mogroside V: 0.18%) of the dried fruit. Therefore, the anti-carcinogenic effect of mogroside V and 11-oxo-mogroside V were expected and examined by the two-stage skin carcinogenesis model using DMBA and TPA. In our experiment, the body weight of the animals was not affected by the treatment with mogroside V and 11-oxo-mogroside V. As shown in Fig. 2, the control group, which received treatment with DMBA and TPA, showed 100% incidence of papillomas within 10 weeks of promotion (Fig. 2A: % of papilloma bearers), and in this group, 3.9, 8.1 and 9.2 papillomas were formed per mouse after 10, 15 and 20 weeks of promotion, respectively (Fig. 2B: the number of papillomas per mouse). On the other hand, in the group treated with DMBA, TPA and 11-oxo-mogroside V, only 26.6 and 53.3% of mice bore papillomas even at 10 and 15 weeks of promotion, respectively, and only 1.0 3.3 and 4.7 papillomas were formed per mouse at 10, 15 and 20 weeks of promotion. From these results, the inhibitory effects of 11-oxo-mogroside V on mouse two-stage carcinogenesis induced by DMBA and TPA were apparently more potent than those of glycyrrhetic acid which has been known as an anti-tumor promoter isolated from licorice root and mogroside V reported in previous paper (in the case of the group treated with glycyrrhetic acid, 50 and 70% of mice bore papillomas and about 4.6 and 6.2 papillomas were formed per mouse at 10 and 15 weeks of promotion, respectively) [4].

Furthermore, the inhibitory effects of mogroside V and 11-oxo-mogroside V on two-stage carcinogenesis of mouse skin initiated by peroxynitrite ($ONOO^-$) and promoted by TPA were investigated for the evaluation of these sweet glycosides as chemopreventive agents. As shown in Fig. 3, in the case of the treatment with peroxynitrite as an initiator, the control group showed more than 85% and 100% incidence of papillomas in less than 10 and 11 weeks of promotion, respectively. The test animals, which had ingested 0.0025% mogroside V for 2 weeks (from 1 week before initiation to 1 week after initiation), took 10 weeks to show even 20% and 20 weeks to show even 80% papilloma formation. This tumor inhibitory effect is also seen as a reduction in the multiplicity of papillomas per mouse over 10 weeks period. In the control group, 3.5, 7.2 and 8.3 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% mogroside V, only 1.2, 2.6 and 4.1 papillomas were formed per mouse after 10, 15 and 20 weeks of promotion, respectively. Furthermore, as shown in Fig. 3, 11-oxo- mogroside V exhibited the similar inhibitory effects to those of mogroside V. As seen, both mogroside V and 11-oxo-mogroside V, taking orally showed the significant inhibitory effects (remarkable delay of papilloma formation and more than 50% reduction in the number of papillomas per mouse over a 20 weeks period) on two-stage skin carcinogenesis initiated by peroxynitrite. These inhibitory effects on the initiation induced by peroxynitrite are also stronger than that of stevioside which had previously reported [4].

From these results of in vivo mouse skin carcinogenesis test, it was concluded that the sweet cucurbitane glycosides, mogroside V and 11-oxo-mogroside V from the fruits of *M. grosvenori*,

inhibited the promotion stages on two stage carcinogenesis induced by TPA. Further, they also inhibited the initiation stages on two stage carcinogenesis induced by peroxynitrite. Therefore, these compounds might be valuable as cancer chemopreventive agents for chemical carcinogenesis, and *M. grosvenori* might be valuable as a source of the chemopreventive agents. And, it was suggested that these natural sweetener will be valuable as a substitute for sucrose in the food ingredient or the food additive. The investigations on details of the inhibitory mechanism of these cucurbitane glycosides on chemical carcinogenesis are being studied.

Acknowledgements

This work was supported in part by the Frontier Research Program of the Ministry of Education, Science and Culture of Japan.

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