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Antitumor Activity of Compounds Isolated from Leaves of *Eriobotrya japonica*

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In a search for possible antitumor agents from natural sources, megastigmane glycosides and polyphenolic constituents isolated from the leaves of *Eriobotrya japonica* (Rosaceae) were found to inhibit the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced activation of Epstein−Barr virus early antigen in Raji cells. Roseoside and procyanidin B-2 were among the active compounds found in an in vitro assay; these compounds were further assessed for antitumor activity in vivo in a two-stage carcinogenesis assay on mouse skin. Roseoside significantly delayed carcinogenesis induced by peroxynitrite (initiator) and TPA (promoter), and its potency was comparable to that of a green tea polyphenol, (−)-epigallocatechin 3-*O*-gallate, in the same assay.

KEYWORDS: ***Eriobotrya japonica*; antitumor activity; Epstein−Barr virus activation; two-stage carcinogenesis; megastigmane glycosides; (−)-epigallocatechin 3-*O*-gallate**

INTRODUCTION

The loquat, *Eriobotrya japonica* Lindl. (Rosaceae), is a small tree native to Japan and China that is widely cultivated for its succulent fruit. Its leaves have been used as a folk medicine for treatment of chronic bronchitis, coughs, phlegm, high fever, and ulcers in Japan and other Asian countries (*1*). A traditional therapy using the leaves in a compress has also been used to treat cancers in Japan. Terpenoids (*2*−*6*) and flavonoids (*7*) have been found in the leaves, and some of these compounds have been reported to be biologically active, exhibiting anti-inflammatory, anti-HIV, or hypoglycemic properties. However, the components responsible for anticarcinogenicity have not been characterized. We recently reported the isolation and characterization of 17 polyphenols, including three new flavonoid glycosides, from the leaves of *E. japonica*, and the potent cytotoxic activity of procyanidin oligomer, a major *E. japonica* polyphenol, against human oral tumor cell lines (*8*). In addition to polyphenolic compounds, we also isolated several triterpenoids and megastigmane glycosides (*9*).

In the present study, individual constituents of *E. japonica* leaves were assessed for their inhibitory effects on the activation of Epstein−Barr virus early antigen (EBV-EA). This assay is a convenient, primary method for identifying possible anti-tumor-promoting agents in vitro. Potent inhibitors of activation in vitro were thereafter studied in vivo in a two-stage carcinogenesis assay on mouse skin. In this assay, 12-dimethylbenz[*a*]anthracene (DMBA) (**7**) or nitric oxide (NO) was used as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was used as a tumor promoter.

MATERIALS AND METHODS

Chemicals and Test Compounds. Cell culture reagents and *n*-butyric acid, TPA, and other reagents for the bioassay were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Peroxynitrite solution, an NO generator, was obtained from Dojindo Laboratories (Kumamoto, Japan).

The test compounds listed in **Table 1** were obtained from the leaves of *E. japonica* (*8*, *9*), and their structures are illustrated in **Figure 1**. Cinchonain IIb (**10**) was among the compounds isolated by repeated chromatography of an ethyl acetate leaf extract over Diaion HP-20 (Mitsubishi Chemicals Co. Ltd., Japan) and Toyopearl HW-40 (Tosoh, Japan) columns in an aqueous methanol solvent. Compound **10** was first isolated from *Cinchona succirubra* (Rubiaceae) (*10*); we report here its first isolation from Rosaceae. (−)-Epigallocatechin 3-*O*-gallate (EGCG) (**12**) was obtained from green tea (*11*).

Short-Term Bioassay for Inhibition of EBV-EA Activation in Vitro. Lymphoblastoid cells carrying the EBV genome (Raji cells derived from Burkitt's lymphoma) were cultured in 10% FBS RPMI-1640 medium (Nissui). Inhibition of EBV-EA activation was assayed using non-virus-producing Raji cells as described previously (*12*). The cells (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 pM) as an inducer, and in the presence and absence of 5 μL of DMSO containing various test compounds. Smears were made from the cell suspensions, and activated cells were detected by indirect immunofluorescence (*13*), using staining

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Table 1. Effect of Test Compounds from *E. japonica* on the Relative Ratio[a] of EBV-EA Activation with Respect to the Positive Control (100%)

compound	concentration (mol ratio/TPA)[b] 1000 (32 μM)	500 (16 μM)	100 (3.2 μM)	10 (0.32 μM)
(6*S*,9*R*)-roseoside (**1**)	0.0 ± 0.3 (60)[c]	20.2 ± 1.2	51.7 ± 1.9	88.2 ± 0.9
(6*S*,9*R*)-vomifoliol-9-*O*-api (1″→6′) glc (**2**)	15.6 ± 0.6 (60)	37.8 ± 1.1	64.0 ± 1.3	100.0 ± 0.2
(6*S*,9*R*)-vomifoliol-9-*O*-xyl (1″→6′) glc (**3**)	10.0 ± 0.2 (60)	30.6 ± 1.3	61.2 ± 1.4	93.4 ± 0.3
(6*R*,9*R*)-3-oxo-α-ionyl-9-*O*-glc (**4**)	8.1 ± 0.5 (60)	24.4 ± 1.5	57.3 ± 1.8	92.5 ± 0.6
eriojaposide A (**5**)	11.8 ± 0.3 (60)	32.4 ± 1.4	63.1 ± 2.1	95.7 ± 0.5
procyanidin B-2 (**6**)	0.0 ± 0.2 (60)	24.5 ± 1.4	75.9 ± 2.2	100.0 ± 0.3
procyanidin C-1 (**7**)	26.5 ± 1.2 (80)	62.1 ± 1.3	88.4 ± 2.0	100.0 ± 0.2
procyanidin oligomer (**8**)	21.4 ± 0.9 (70)	57.9 ± 1.5	89.2 ± 1.9	100.0 ± 0.2
cinchonain Id 7-*O*-glc (**9**)	21.3 ± 0.8 (60)	39.9 ± 1.1	69.1 ± 1.7	100.0 ± 0.4
cinchonain IIb (**10**)	19.5 ± 1.7 (60)	38.6 ± 1.2	68.0 ± 1.7	100.0 ± 0.4
(2*S*)-naringenin 8-*C*-Rha (1″→2′) glc (**11**)	10.6 ± 0.4 (80)	34.7 ± 1.3	63.9 ± 2.1	100.0 ± 0.3
(−)-epigallocatechin 3-*O*-gallate (**12**)	6.4 ± 0.8 (70)	34.9 ± 1.3	68.1 ± 2.1	87.7 ± 0.9

[a] Values represent percentages relative to the positive control value (100%). [b] TPA concentration was 20 ng (32 pM)/mL. [c] Values in parentheses represent the percentage of viable Raji cells; unless otherwise stated, the viability was >80%.

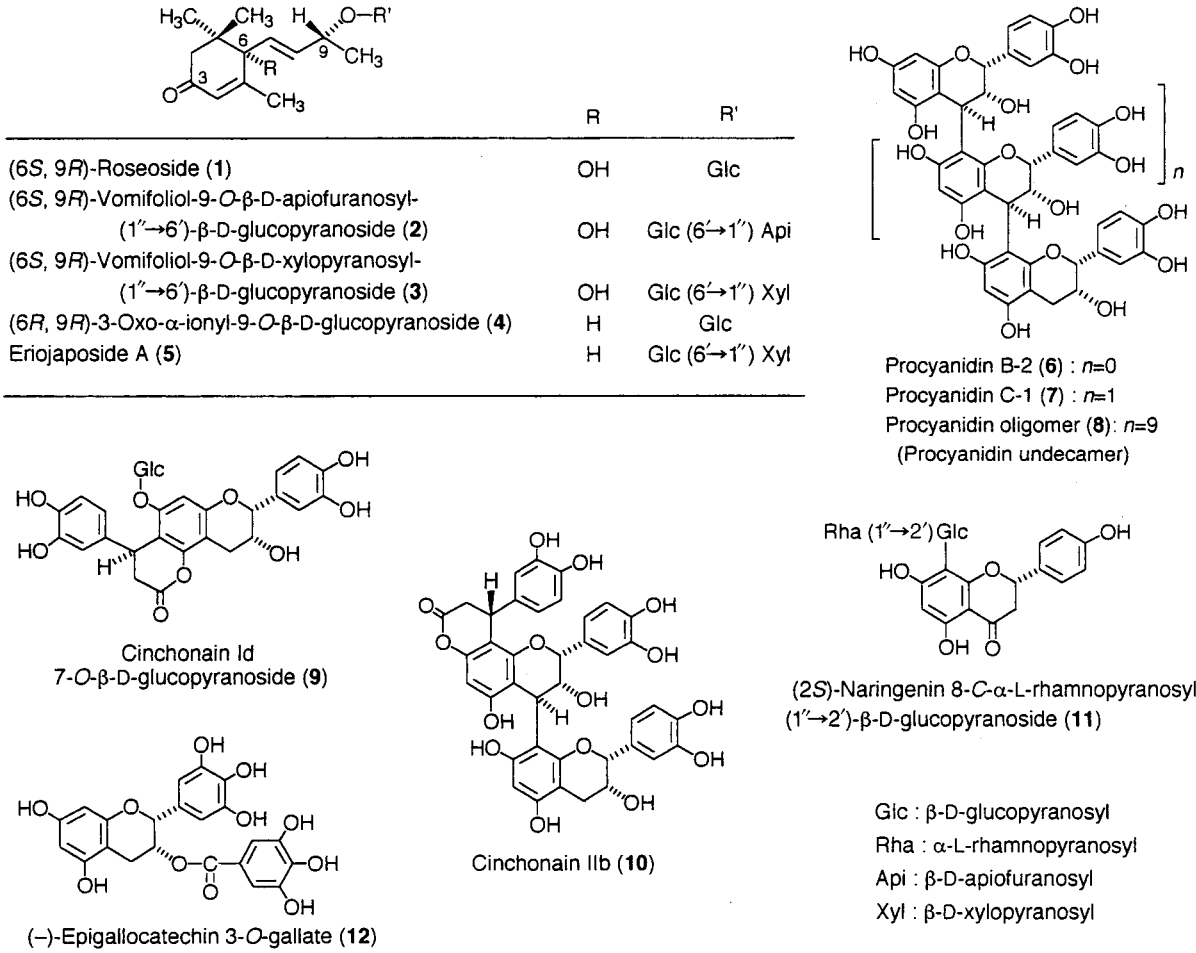

Figure 1. Structures of megastigmane glycosides, procyanidins, and flavonoids from *E. japonica*.

with EBV-EA-positive serum from nasopharyngeal carcinoma (NPC) patients as the criterion for activation. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) present was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compounds was expressed relative to the results of the control experiment, which averaged ~35%. The viability of the treated Raji cells was assayed according to the trypan-blue exclusion staining method.

Two-Stage Carcinogenesis Test on Mouse Skin. For stage 1, specific-pathogen-free 6-week-old female ICR mice were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were housed in a temperature-controlled room at 24 ± 2 °C at five per polycarbonate cage and were given food and water ad libitum throughout the experiment. Animals were divided into three experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were topically treated with 100 μg of DMBA (0.1 mL of a 390 nM solution in acetone) as an initiating treatment. One week after the initiation, papilloma formation was promoted by application twice a week of 1 μg of TPA (0.1 mL of a 1.7 nM solution in acetone) to the skin. One hour before each TPA treatment, the mice were treated with 0.1 mL of the test compounds (85 nM in acetone). The incidence and numbers of papillomas were examined weekly over the course of 20 weeks.

In stage 2, a peroxynitrite−TPA carcinogenesis test was performed. The dorsal regions of 30 6-week-old female SENCAR mice were shaved with an electric clipper. One day later, 0.1 mL of a 390 nM peroxynitrite

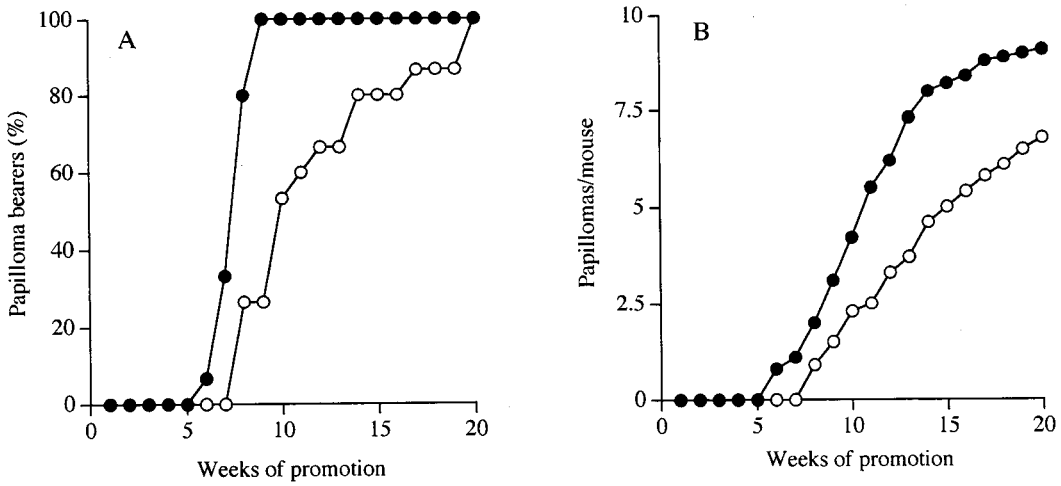

Figure 2. Inhibition of TPA-induced tumor promotion by multiple applications of procyanidin B-2 (**6**): (A) percentage of mice bearing papillomas; (B) average number of papillomas per mouse [●, control, TPA alone; ○, TPA + **6** (85 nM)]. Papillomas per mouse after treatment with **6** differed significantly from the positive control at 20 weeks after promotion ($P < 0.01$). Tumor formation was initiated with DMBA (390 nM) and promoted with 1.7 nM of TPA given twice weekly starting 1 week after initiation.

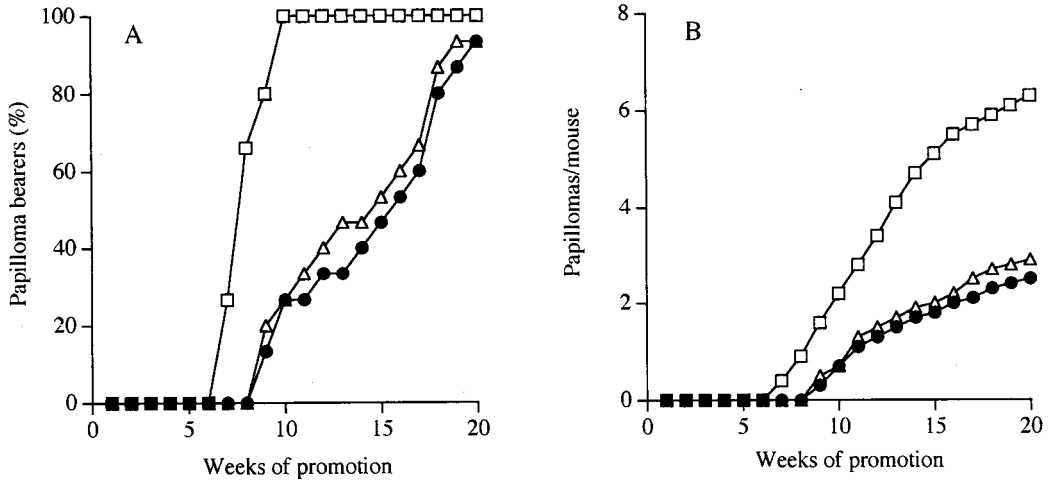

Figure 3. Inhibition of TPA-induced tumor promotion by multiple applications of roseoside (**1**) and (–)-epigallocatechin 3-*O*-gallate (**12**): (A) percentage of mice bearing papillomas; (B) average number of papillomas per mouse [□, control, TPA alone; △, TPA + **1** (0.0025%); ●, TPA + **12** (0.0025%)]. Tumor formation was initiated with peroxynitrite (390 nM) and promoted with TPA (1.7 nM) given twice weekly starting 1 week after initiation. Papillomas per mouse after treatment with test compounds differed significantly from the positive control at 20 weeks after promotion ($P < 0.01$).

solution in 1 mM NaOH was topically applied as an initiation treatment. This treatment was repeated daily for one week. The test compounds were orally administered to 15 of the mice at 0.0025% in their drinking water, both before and after the week of peroxynitrite treatment, whereas the other 15 mice received drinking water only as a control. One week after initiation treatments were terminated, all mice were treated topically with 0.1 mL of 1.7 nM TPA in acetone twice a week. Mice were examined once a week for tumors, and tumors of at least 1-mm diameter were recorded. Both the percentage of tumor-bearing mice relative to the control group and the average number of tumors per mouse were determined for each test compound.

RESULTS AND DISCUSSION

Five megastigmane glycosides (**1**−**5**), three procyanidins (**6**−**8**), and three flavonoids (**9**−**11**) isolated from *E. japonica* leaves were first assessed for anti-tumor-promoting activity in a short-term TPA-induced EBV-EA activation assay. The results are shown in **Table 1**.

All of the test compounds except trimeric and oligomeric procyanidin C-1 significantly inhibited (60−80%) EBV-EA activation at a concentration of 500 M ratio/TPA (16 μM) without exhibiting cytotoxicity. Their potencies were comparable to or stronger than that of the positive control EGCG (**12**), a green tea polyphenol that has been well characterized as an anti-tumor-promoting agent (*14*). Notably, an increase in the molecular size of the procyanidins correlated with reduced potency, and the inhibitory effect of procyanidin B-2 (**6**) (75.5%) was greater than that of the other dimers, procyanidins B-1, B-4, and B-5 (34−58%), at the same concentration (*15*). In the series of megastigmane glycosides tested, the glucose-glycosylated compounds (**1** and **4**) were more potent than their disaccharide-glycosylated analogues (**2**, **3**, and **5**). Potency appeared to be unaffected by the presence or absence of a hydroxyl group at C-6 of the megastigmane nucleus, however.

Procyanidin B-2 (**6**), a potent inhibitor of EBV-EA activation, was examined for its effect on two-stage mouse skin carcinogenesis using DMBA as an initiator and TPA as a promoter. In the control group, 100% of the mice bore papillomas at 9 weeks after TPA treatment (**Figure 2A**), whereas only 25 and 80% of the mice treated with **6** bore papillomas at 9 and 16 weeks, respectively. At week 20, the average number of papillomas per mouse was reduced to 75% of the control value (**Figure 2B**).

Roseoside (**1**), the most potent inhibitor of EBV-EA activation, could not be examined for its anti-tumor-promoting effect in vivo according to the above method used for procyanidin B-2 (**6**) because an insufficient amount of compound was available. Therefore, roseoside was subjected to a recently developed, alternative in vivo assay, in which nitric oxide (NO) is used as an initiator (*16*). NO, a bioactive gaseous radical, has been recognized as an important factor in bacterial, parasitic, and viral infections and also in several steps of carcinogenesis including mutagenicity (*17*−*19*). In the two-stage mouse skin carcinogenesis experiment, NO generated from peroxynitrite was demonstrated to increase papillomas when it was used with the promoter TPA, indicating that NO acts as an initiator (**Figure 3A**) (*16*). Hence, the inhibitory effect on tumor initiation was estimated using an assay in which the test compound was administered in drinking water only at the peroxynitrite initiation step. This method has the advantage that only a small amount of sample is used ($\sim^1/_{10}$ of the amount for DMBA/TPA-induced carcinogenesis). EGCG was found to be an effective anti-initiating agent for this assay (**Figure 3**). At week 20 after initiation, the average number of papillomas per mouse was ~40% of the control value. Notably, roseoside (**1**) had a potent anti-initiating effect comparable to that of EGCG at the same concentration (**Figure 3**), although the mechanism of this effect remains unclear. This paper describes the first evidence of an antitumorigenic effect for this megastigmane glycoside.

In conclusion, this study found that compounds possessing anti-tumor-promoting and anti-tumor-initiating properties are present in the leaves of *E. japonica*, in addition to a procyanidin oligomer that has selective cytotoxicity against human oral tumor cell lines (*8*). Thus, *E. japonica* is promising as a potential source of agents for chemoprevention of cancers.

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