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PRODUCTION OF ANTI-TUMOR-PROMOTING IRIDOID GLUCOSIDES IN *GENIPA AMERICANA* AND ITS CELL CULTURES

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ABSTRACT.—The *Genipa americana* plant contains geniposide [**3**] and geniposidic acid [**2**] in the fruits and geniposidic acid [**2**] in the leaves. On callus induction, the plant produces tarennoside [**1**], geniposidic acid [**2**], and gardenoside [**4**] in high levels. The leaves of *Ge. americana* plants redifferentiated from the callus tissues produce **2**.

Genipa americana L. is a rubiaceous plant native to tropical Central and South America. The elliptically shaped fruit of the plant is edible and popular as a source of beverages. The white flesh turns yellow to bluish-purple and finally to jet-black on exposure to the air. The plant was introduced in Java in 1913 (1,2).

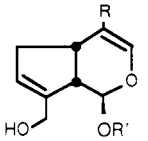
Genipin [**5**] was isolated from the ripe fruit (3) and the structure was elucidated (4). Two cyclopentanoid monoterpenes, genipic acid and genipinic acid, were isolated from Puerto Rican *Ge. americana* fruit. Genipin [**5**], however, was not obtained from that source (5). Subsequently, geniposidic acid [**2**] was isolated as a powder from the leaves of *Ge. americana* collected in Darien, Panama (6).

Gardenia jasminoides Ellis, a related rubiaceous plant growing throughout subtropical to temperate East Asia from Viet Nam to the southern part of Japan (7), also produces geniposidic acid [**2**], geniposide [**3**], a glucoside of genipin [**5**], and genipin gentiobioside along with gardenoside [**4**] (8,9). Later, tarennoside [**1**] (10), the main iridoid glucoside of Okinawan *Tarenna gracilipes* (Hey.) Ohwi, and **2**, **3**, and **4** were obtained from the cultured cells of *Ga. jasminoides* (11). Administration of the isotopically labeled putative precursors to the *Ga. jasminoides* cell cultures established the biosynthetic sequence **1**↦**2**↦**3**↦**4** starting from mevalonic acid (12,13).

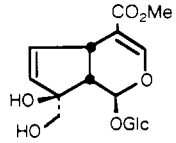
This paper describes the isolation of geniposidic acid [**2**] and geniposide [**3**] from the fresh fruits of *Ge. americana*, production of tarennoside [**1**], geniposidic acid [**2**], and gardenoside [**4**] in the callus and cell suspension cultures of *Ge. americana*, and the anti-tumor-promoting activity of the above-described iridoids, including genipin [**5**].

RESULTS AND DISCUSSION

Fresh fruits of *Ge. americana* collected at Bogor Botanic Garden, Indonesia in October 1983, were extracted with hot MeOH immediately after being cut into



1 R = CHO, R′ = glc
2 R = CO_2H, R′ = glc
3 R = CO_2Me, R′ = glc
5 R = CO_2Me, R′ = H



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glc = β-D-glucopyranosyl

slices. The MeOH extracts, after conventional workup including Si gel cc and preparative tlc, gave geniposide [**3**] as the main iridoid glucoside and a small amount of geniposidic acid [**2**]. After prolonged standing, the slices gradually colored bluish-purple. The MeOH extract of the colored slices gave genipin [**5**] in addition to **2** and **3**. These results suggest that genipin [**5**] is formed through the hydrolysis of **3** during the course of ripening or storage.

Callus tissues induced from the seedlings or the leaves of *Ge. americana* on the Linsmaier-Skoog medium supplemented with 2,4-D were subcultured on the Murashige-Skoog medium supplemented with IAA and kinetin. The method of selection of iridoid-producing cells was analogous to that used previously for *Ga. jasminoides* (11). The callus and cell suspension cultures of *Ge. americana* produce tarennoside [**1**], geniposidic acid [**2**], and gardenoside [**4**] in the same levels as in the case of *Ga. jasminoides* cell cultures. The leaves of intact plants redifferentiated from the callus tissues through embryogenesis produce only **2**.

The tumor promotion inhibiting activity of the above-described iridoids against the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus (EBV) activation in a nonproducer Raji cell line was examined by a short-term in vitro assay (14,15). TPA was found to act on protein kinase C activation in this assay system (H. Tokuda, unpublished results). Of the four iridoid glucosides, tarennoside [**1**] is the most active, and the activity decreases with the progress of the metabolism (12,13). Although the activity of geniposide [**3**] is lower than that of geniposidic acid [**2**], its aglycone genipin [**5**] is remarkably active (Table 1). Genipin [**5**] not only has a higher inhibitory activity against the TPA-induced EBV activation than that of retinoic acid (16), it also has an almost negligible cell toxicity with respect to that of retinoic acid.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. Uv spectra were determined with a Hitachi 200-20 spectrometer in MeOH solution, ir spectra (KBr) with a Shimadzu IR-435 spectrophotometer, and ^{1}H-nmr spectra in CD_3OD (iridoid glucosides) or $CDCl_3$ (genipin) with a JEOL-JNM-FX-200-FT spectrometer (200 MHz) with TMS as the internal standard. Optical rotations were measured in MeOH on a Jasco DIP-181 digital polarimeter. Si gel 60

TABLE 1. Inhibitory Effects of *Genipa americana* Iridoids Against 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr Virus Early Antigen Activation.[a]

Compound	Concentration (molar ratio/TPA)			
	1000	500	100	10
Tarennoside [**1**]	30.5 ± 4.5[b] (80.0)[c]	72.8 ± 2.4	88.1 ± 3.0	100.0 ± 0
Geniposidic acid [**2**]	34.8 ± 3.3 (80.0)	80.5 ± 3.1	91.8 ± 3.5	100.0 ± 0
Geniposide [**3**]	62.4 ± 3.0 (80.0)	91.0 ± 2.4	100.0 ± 0.8	100.0 ± 0
Gardenoside [**4**]	92.6 ± 1.8 (80.0)	100.0 ± 1.1	100.0 ± 0	100.0 ± 0
Genipin [**5**]	11.5 ± 2.4 (80.0)	44.2 ± 3.9	64.2 ± 3.1	100.0 ± 1.5
1 + **2** + **3** + **4** + **5** (molar ratio 1:1:1:1:1)	26.7 ± 3.2 (70.0)		70.0 ± 2.5	100.0 ± 0
(retinoic acid)	30.0 ± 5.0 (10.0		67.0 ± 3.3 (40.0)	

[a]TPA (32 pmol), 100 = positive control.

[b]Values are % control ±SD.

[c]Values in parentheses represent the viability % measured through Trypan Blue staining followed by counting of surviving Raji cells 48 h after the concomittant treatment of the cells with TPA, *n*-butyrate, and test substances in a 0.25% phosphate buffer solution (pH 7.2).

(Merck) was used for cc, and Si gel 60 GF_{254} and PF_{254} (Merck) were used for tlc.

PLANT MATERIAL.—The leaves and fruits of *Ge. americana* native to West Indies were collected at Bogor Botanic Garden, Indonesia in October 1983. The registration numbers of the *Ge. americana* trees are V.E. 27-27a and 28-28a. Voucher specimens have been deposited in the herbarium of the Department of Botany, Faculty of Science, Kyoto University (KYO).

CALLUS INDUCTION AND CULTURE CONDITIONS.—*Ge. americana* callus tissues were induced from the seedlings or the leaves on the Linsmaier-Skoog medium supplemented with 2,4-D (10^{-5} M). According to the method applied to *Ga. jasminoides* (11), iridoid-producing cell lines were selected during subculture on Murashige-Skoog medium supplemented with IAA (2×10^{-5} M) and kinetin (10^{-5} M), and this medium was used to grow callus cultures. The sucrose content was always 3%. The callus tissues were cultivated at 25° in the dark or under illumination with a white light (ca. 6000 lux). Plants redifferentiated through embryogenesis were transferred to the hormone-free Murashige-Skoog medium and cultured under illumination of white light (ca. 6000 lux). After substantial root growth (ca. 1 cm), seedlings were transferred to pots containing vermiculite and sand. They were grown to a height of 1.5 m in a greenhouse at the Faculty of Pharmaceutical Sciences, Kyoto University over the course of 7 years. Seedlings were also obtained through the culture of apical meristems.

ISOLATION OF IRIDOIDS FROM *GE. AMERICANA* FRUITS, LEAVES OR CULTURED CELLS.—Extraction with MeOH under reflux for 30 min (4 times) followed by concentration in vacuo of the combined extracts gave a syrupy residue. This was dissolved in H_2O and filtered. The filtrate was washed with $CHCl_3$. The aqueous layer was concentrated in vacuo, applied to a charcoal column, and eluted with H_2O and aqueous MeOH with increasing MeOH content. Fractions indicating the positive mineral acid test (17) were combined and concentrated in vacuo to give a pale yellow solid residue, which was subjected to preparative tlc with $CHCl_3$-MeOH (4:1). Extraction of the tlc bands with $CHCl_3$-MeOH (9:1) gave tarennoside [**1**], geniposidic acid [**2**], geniposide [**3**], or gardenoside [**4**].

IRIDOID GLUCOSIDES.—Tarennoside [**1**] was obtained as a white powder, $[\alpha]^{25}D$ +20.0° (c = 0.5, MeOH). The spectral data (uv, ir, and ^1H nmr) were identical with those of an authentic sample. The yield of **1** from the callus tissues was 0.3% on a dry wt basis. Geniposidic acid [**2**] was recrystallized from MeOH to give colorless needles, mp 133–136°, $[\alpha]^{25}D$ +10.0 (c = 0.5, MeOH). This compound was identified with an authentic crystalline sample obtained from *Ga. jasminoides* cell cultures (S. Ueda, unpublished results) by mixed mp and comparisons of spectral data. The yields of **2** from the fruits, leaves, and callus tissues on a dry wt basis were 0.01, 0.02, and 0.04%, respectively. Geniposide [**3**] was recrystallized from EtOH to give colorless needles, mp 165°. The optical rotation and the spectral data were identical with those of an authentic sample. Gardenoside [**4**] was obtained as a white powder, $[\alpha]^{25}D$ −90.4° (c = 1.0, MeOH). The spectral data were identical with those of an authentic sample. The content of **3** in the fresh fruits and that of **4** in the callus tissues were 0.2 and 0.15%, respectively.

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