Inhibitory Effects of Leaves of Guava (*Psidium guajava*) on TPA-Induced Inflammation and Tumor Promotion in Two-Stage Carcinogenesis in Mouse Skin

Ken Yasukawa[1,*] and Tomohito Kakegawa[2]

[1]*School of Pharmacy, Nihon University, 7-7-1, Narashinodai, Chiba 274-8555, Japan*

[2]*Faculty of Pharmaceutical Sciences, Josai International University, 1 Gumyo, Togane, Chba 283-8555, Japan*

Abstract: Cancer prevention offers the most cost-effective long-term health strategy. The methanol extract of the leaves of guava (*Psidium guajava*) exhibits marked antitumor activity in an *in vivo* two-stage carcinogenesis test in mice using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and 12-*O*-tetradecanoylphorbol-13- acetate (TPA) as a promoter. From the active fraction of the methanol extract, five triterpene acids, uvaol (**1**), ursolic acid (**2**), corosolic acid (**3**), asiatic aci (**4**), and oleanolic acid d (**5**), were isolated and identified. These compounds were evaluated for their inhibitory effects on TPA-induced inflammation (1 μg/ear) in mice, and showed marked anti-inflammatory effects, with a 50% inhibitory dose of 117–657 nmol/ear. The leaves of guava may therefore be effective for cancer prevention.

Keywords: Guava, *Psidium guajava*, antitumor promotion, anti-inflammation, cancer prevention.

INTRODUCTION

In the field of public health, treatment of cancers is one of the most important problems at present. We have isolated and identified numerous compounds for cancer prevention from numerous plants and fungi [1,2]. Among these, we have prepared several reports on triterpenes [3,4].

Guava (*Psidium guajava* L.; Myrtaceae) is an important food crop and medicinal plant in tropical and subtropical countries, and is widely used in foods and folk medicine around the world [5]. More recent ethnopharmacological studies have shown that guava is used in many parts of the world as an anti-inflammatory for the treatment of a number of conditions, e.g., diabetes, hypertension, caries, wounds, pain and fever [6]. In Japan, guava leaves are used as a supplement for hypertension, hypercholesterolemia and diabetes. The chemical constituents of *Psidium guajava* reportedly include flavonoids, tannins, monoterpenes, sesquiterpenes, triterpenes and carotenoids [5].

In the present study, the mthanol extract from the guava leaves was found to inhibit TPA-induced tumor promotion during two-stage carcinogenesis in mouse skin. Five triterpenoids were subsequently isolated from the methanol extract of guava leaves. The 50% inhibitory doses of these compounds for TPA-induced inflammatory ear edema were 117 - 657 nmol/ear.

*Address correspondence to this author at the School of Pharmacy, Nihon University, 7-7-1, Narashinodai, Funabashi, Chiba 274-8555, Japan;
Tel/Fax: +81 47 465 1107; E-mail: yasukawa.ken@nihon-u.ac.jp, yasukawa.ken@nihon-u.ne.jp

MATERIALS AND METHODS

General Experimental Procedures

^{1}H- and ^{13}C-NMR spectra were obtained with a JEOL ECX-500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer (Japan), and chemical shifts are shown as values relative to tetramethylsilane as an internal standard. Mass spectra were measured with a JEOL JMS-GC mass spectrometer (Japan) at an ionization voltage of 70 eV. High performance liquid chromatography (HPLC) was performed on a C_{18} silica column (Pegasil ODS column, 25 cm $\times$ 20 mm i.d.; Shenshu Scientific Co., Tokyo, Japan) with methanol as a mobile phase (flow rate 2 ml/min).

Chemicals

TPA was purchased from Chemicals for Cancer Research, Inc. (Minnesota, MN). 7,12-Dimethylbenz[*a*] anthracene, dimethyl sulfoxide, indomethacin and hydrocortisone were obtained from Sigma Chemical Co. (St. Louis, MO). Methanol, *n*-butyl alcohol, etyl acetate, *n*-hexane, chloroform and acetone were obtai-ned from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

Materials

Leaves of guava (*Psidium guajava* L.) were obtained from Kinokuniya Kan-Yakkyoku in 2007 in Tokyo, Japan. Voucher specimens “SM0702” were deposited at the School of Pharmacy, Nihon University.

Extraction and Isolation

Dry leaves of guava (1.12 kg) were subjected to extraction five times for 3 days with methanol at room

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temperature to give an extract (87.4 g), which was partitioned between ethyl acetate-water (1:1) to yield an ethyl acetate extract (33.4 g). The ethyl acetate extract was partitioned between *n*-hexane–methanol–water (19:19:2), which afforded *n*-hexane (24.7 g) and methanol–water (8.71 g) extracts, respectively. The water solution was partitioned between *n*-butyl alcohol-water (1:1), yielding an *n*-butyl alcohol extract (16.4 g) and an water extract (37.6 g), respectively.

The methanol-water extract (8.5 g) was subjected to column chromatography (CC) on Sephadex LH-20 (Parmacia LKB, Uppsala, Sweden) using Chloroform-methanol (1:1) to obtain eight fractions: fraction 1 (245 mg), fraction 2 (3.50 g), fraction 3 (988 mg), fraction 4 (141.0 mg), fraction 5 (182 mg), fraction 6 (646 mg), fraction 7 (428 mg) and fraction 8 (158.0 mg). Fraction 3 (960 mg) was further separated on Pegasil ODS (Shenshu Scientific Co., Tokyo Japan) using methanol to isolate **2** (24.3 mg), **3** (16.4 mg), **4** (19.2 mg) and **5** (29.3 mg). Fraction 4 (130 mg) was then purified by reversed-phase preparative HPLC (Pegasil ODS, methanol) to isolate **1** (20.6 mg).

Identification

Identification of **1** [7], **2** [8], **3** [9], **4** [8] and **5** [7] was performed by spectral comparison with literature data. Full details of the isolation and identification, as well as the spectral data, are available on request from the corresponding author.

Animals

Experiments were performed in accordance with the Guidelines of the Institutional Animal Care and Use Committee of the School of Pharmacy, Nihon University, Chiba, Japan. Female ICR mice (age, 7 weeks) were purchased from Japan SLC Inc. (Shizuoka, Japan), and were housed in an air-conditioned specific pathogen-free room (24 ± 2°C) with lights on from 08:00 to 20:00. Food and water were available *ad libitum*.

Assay of TPA-Induced Inflammation in Mice

TPA (1 μg) dissolved in acetone (20 μl) was applied to the right ear of ICR mice using a micropipette. A volume of 10 μl was delivered to both the inner and outer surfaces of the ear. The sample (0.01–1.0 mg/ear) or vehicle, methanol-chloroform-water (2:1:1; 20 μl) or methanol-chloroform (1:1; 20 μl) as a control, was applied topically about 30 min before TPA treatment. For ear thickness determination, a pocket thickness gauge (Mitsutoyo Co. Ltd., Tokyo, Japan) with a range of 0–9 mm (graduated at 0.01-mm intervals and modified so that the contact surface area was increased, thus reducing tension) was applied to the tip of the ear.

Ear thickness was determined before TPA treatment (*a*).

Edema was measured at 6 h after TPA treatment (*b*: TPA alone; *b'*: TPA with sample). The following values were then calculated:

Edema A: edema induced by TPA alone ($b - a$).

Edema B: edema induced by TPA plus sample ($b' - a$).

Inhibitory ratio (%) = [(edema A – edema B)/edema A] $\times$ 100.

Each value was the mean of individual determinations from four to five mice.

Two-Stage Carcinogenesis Experiment

The backs of mice (age, 7 weeks) were shaved with electric clippers. Initiation was accomplished by single topical application of 50 μg of DMBA. Promotion with 1 μg TPA, applied twice weekly, was started 1 week after initiation. The methanol extract of the guava leaves (1.0 mg/mouse), or its vehicle, acetone-dimethylsulfoxide-water (8:1:1; 100 μl), was applied topically 30 min before each TPA treatment. DMBA and TPA were dissolved in acetone, and were applied to the shaved area in a volume of 100 μl using a micropipette. The back of each animal was shaved once a week to remove hair. The number and diameter of skin tumors were measured every week, and the experiment was continued for 20 weeks. Experimental and control groups each consisted of 15 mice.

Statistical Analysis

The 50% inhibitory dose (ID_{50}) values and their 95% confidence intervals (95% CI) were obtained by nonlinear regression using the GraphPad program 5.0 (Intuitive Software for Science, San Diego, CA). Differences between experimental groups were compared by Student's *t*-test and Mann-Whitney *U* exact test.

RESULTS AND DISCUSSION

As can be seen in Table **1**, the extracts from guava leaves inhibited TPA-induced inflammation in mice. The inhibitory effects of the methanol extract of guava

Table 1: Inhibitory Effects of Guava Leaves on TPA-Induced Inflammatory Ear Edema

Sample	I.R.
Methanol extract (1.0 mg/ear)	75**
n-Hexane extract of methanol extract (1.0 mg/ear)	60**
Methanol-water extract of methanol extract (1.0 mg/ear)	89**
n-Butyl alcohol extract of methanol extract (1.0 mg/ear)	33**
Water extract of methanol extract (1.0 mg/ear)	9
Fraction 1 from methanol-water extract of methanol extract (0.5 mg/ear)	29*
Fraction 2 from methanol-water extract of methanol extract (0.5 mg/ear)	45**
Fraction 3 from methanol-water extract of methanol extract (0.5 mg/ear)	89**
Fraction 4 from methanol-water extract of methanol extract (0.5 mg/ear)	62**
Fraction 5 from methanol-water extract of methanol extract (0.5 mg/ear)	41**
Fraction 6 from methanol-water extract of methanol extract (0.5 mg/ear)	21*
Fraction 7 from methanol-water extract of methanol extract (0.5 mg/ear)	15
Fraction 8 from methanol-water extract of methanol extract (0.5 mg/ear)	6

I.R.: Inhibitory ratio. *$p < 0.05$, **$p < 0.01$.

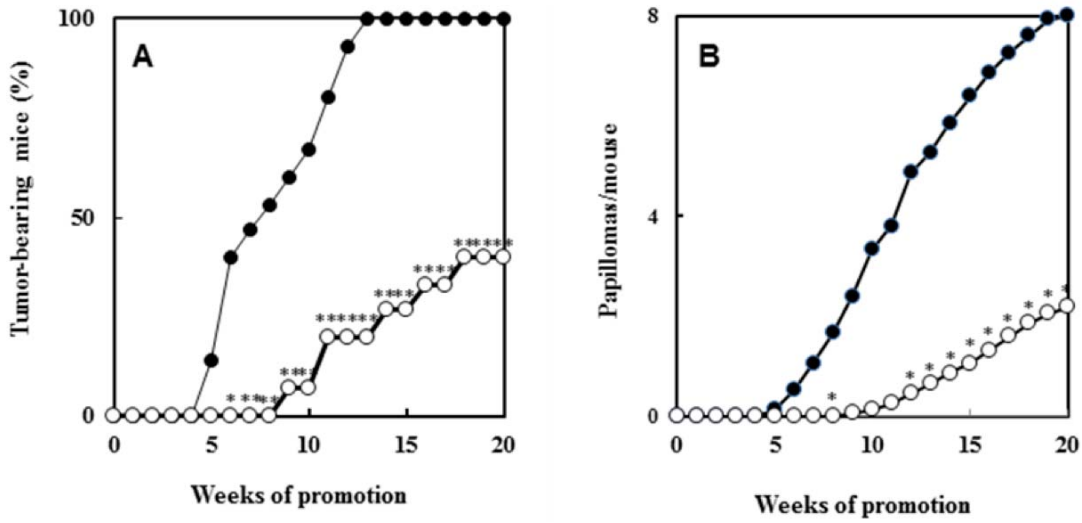


Figure 1: Inhibitory effects of methanol extract of guava leaves on tumor promotion of skin papillomas by TPA in DMBA-initiated mice.

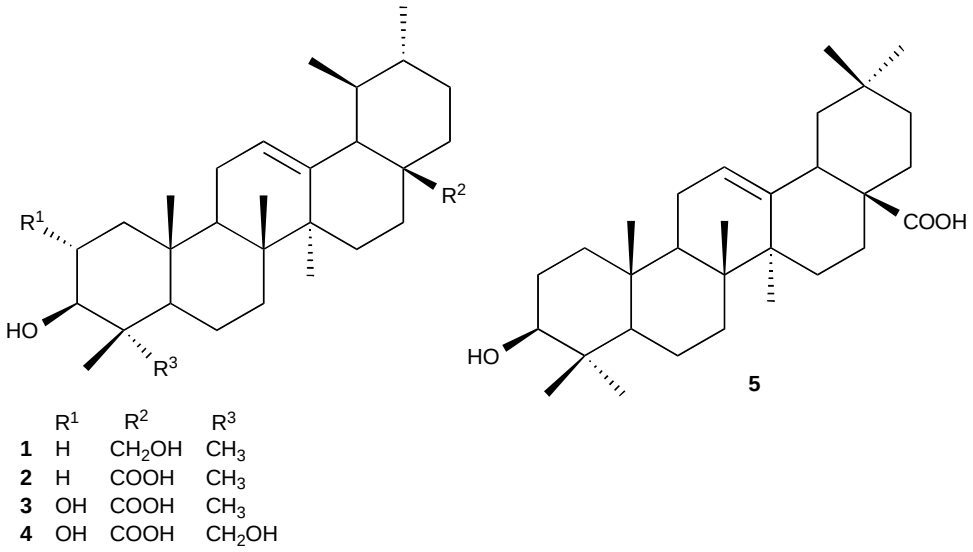
From 1 week after initiation with a single topical application of 50 μg of DMBA, 1 μg of TPA was applied twice weekly. Topical application of methanol extract (1 mg) and vehicle was performed 30 min before each TPA treatment. Data are expressed as the percentage of mice bearing papillomas (**A**) and as the average number of papillomas per mouse (**B**). ●, + TPA with vehicle alone; ○, + TPA with methanol extract of guava leaves. The treated group was determined to be statistically different from the control group by Mann-Whitney *U* exact test (**A**) and by Student's *t*-test (**B**). * $p < 0.05$ and ** $p < 0.01$.

leaves in a two-stage carcinogenesis test on mouse skin using DMBA as an initiator and TPA as a tumor promoter were then investigated. Figure **1A** illustrates the time course of skin tumor formation in the groups treated with DMBA plus TPA, with or without the methanol extract of guava leaves. The first tumor appeared at week 5 in the group treated with DMBA plus TPA and all 15 mice had tumors at week 13. In the group treated with DMBA plus TPA and methanol extract of the leaves of guava, the first tumor appeared at week 9. The percentage of tumor-bearing mice treated with DMBA plus TPA and methanol extract of guava leaves was 40% at week 20. Figure **1B** shows the average number of tumors per mouse. The group treated with DMBA plus TPA produced 8.0 tumors per mouse at week 20; the group treated with DMBA plus TPA and methanol extract of the leaves of guava had 2.2 tumors per mouse. Treatment with methanol extract

Table 2: Inhibitory Effects of Components from Guava Leaves on TPA-Induced Inflammatory Ear Edema

Component	ID_{50}	95% CI
	(nmol/ear)	
Uvaol (**1**)	243	164 - 358
Ursolic acid (**2**)	219	131 - 328
Corosolic acid (**3**)	190	148 – 222
Asiatic acid (**4**)	117	79.4 – 172
Oleanolic acid (**5**)	657	526 - 788
Indomethacin[a]	908	755 – 1092
Hydrocortisone[a]	69.1	64.3 – 75.4

ID_{50}: 50% inhibitory dose. 95% CI: 95% confidence intervals. [a]Reference compound.



	R^1	R^2	R^3
1	H	CH_2OH	CH_3
2	H	COOH	CH_3
3	OH	COOH	CH_3
4	OH	COOH	CH_2OH

Figure 2: Chemical structures of triterpenoids from guava leaves.

of guava leaves caused a 73% reduction in the average number of tumors per mouse at week 20. By comparison with methanol extracts of supplemental foods, guava leaves showed similar activity as mesima mushroom [10], chaga mushroom [11], galangal [12], Brazilian propolis [13], gymnema [14] and seabuckthorn [15].

Active components (**1**–**5**) were then isolated from the active fractions of the methanol extract. The isolated compounds showed inhibitory activity against TPA-induced ear inflammatory edema. As shown in Table **2**, the ID_{50} of these compounds on TPA-induced inflammation was 117 - 657 nmol/ear, respectively. In comparison with standard drugs, all compounds except oleanolic acid (**5**) showed a stronger depression effect than indomethacin, an anti-inflammatory drug. The inhibitory effects against TPA-induced inflammation have been demonstrated to closely parallel those of the inhibition of tumor promotion in two-stage carcinogenesis initiated by DMBA and then by TPA, a well-known tumor promoter, in a mouse skin model [16]; thus, these triterpenes from the guava leaf might be expected to possess a high antitumor-promoting effect in the same animal model. Many triterpenoids have been found to inhibit tumor promotion in two-stage carcinogenesis in mouse skin [3,4]. Ursolic acid has been found to inhibit tumor promotion in two-stage carcinogenesis in mouse skin [17,18]. The effects of ursolic acid are propagated through the suppression of cyclooxygenase-2 [19,20], tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) [21], matrix metalloproteinase 9 [22] and apoptosis [23]. TNF-α plays an important role in tumor promotion in multiple stages of carcinogenesis [24].

In conclusion, this is the first report to find that triterpenes from guava leaves inhibited tumor promoter-induced inflammation in mice. Furthermore, the methanol extract of guava leaves inhibited tumor promotion by TPA following initiation with DMBA in ICR mouse skin. The active components (uvaol, ursolic, corosolic, asiatic and oleanolic acids (**1**–**5**)) were isolated from the active fraction of the methanol extract of guava leaves.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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