

Inhibitory Effects of Brazilian Propolis on Tumor Promotion in Two-Stage Mouse Skin Carcinogenesis

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Abstract: Propolis is produced by honeybees and has many biological properties, including immunomodulatory, anti-inflammatory, anti-oxidant, anti-bacterial, anti-viral and anti-cancer actions. Five ethanol extracts of Brazilian propolis were tested for inhibition of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. The ethanol extract of Brazilian propolis AF-08 markedly inhibited TPA-induced inflammatory ear edema in mice. This extract suppressed tumor promotion by TPA following initiation with 7,12-dimethylbenz[a]anthracene (DMBA) in mouse skin. Moronic acid, a major component of Brazilian propolis AF-08, markedly inhibited two-stage carcinogenesis by DMBA plus TPA. These results suggest that Brazilian propolis AF-08 contributes to the prevention of cancer.

Keywords: Propolis, moronic acid, cancer chemoprevention, tumor promotion, two-stage carcinogenesis.

1. INTRODUCTION

Complementary and alternative medicine (CAM) plays an important role in the prevention of cancer. Strategies used in CAM for cancer prevention involve the use of several types of propolis, as propolis has been reported to possess anti-tumor activity [1]. Our previous studies have illustrated that supplementation with mushrooms such as reishi [2] and meshima-kobu (hardwood trunk rot) [3], as well as galangal [4] and chlorella [5], inhibit tumor promotion in two-stage mouse skin carcinogenesis.

Propolis is produced by honeybees and is used both as a dietary supplement worldwide and extensively in folk medicine for its anti-inflammatory [6], anti-oxidant [6], immunomodulatory [7] and anti-cancer [8] effects. Bees around the globe collect and utilize resins as propolis for a number of purposes, including sealing cracks in the nest, creating smooth surfaces for comb attachment, entombing parasites and predators and reducing in-hive microbes [9].

In this paper, we report that topical application of Brazilian propolis AF-08 (BP-AF-08) inhibits tumor promotion in a two-stage mouse skin carcinogenesis test, using 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as a promoter. In addition, the major component

of BP-AF-08, moronic acid, markedly inhibited tumor promotion in two-stage carcinogenesis in mouse skin.

2. MATERIALS AND METHODS

2.1. Materials

Brazilian propolis was obtained from Amazon Food Co. Ltd. (Tokyo, Japan). The voucher specimens (AF-06, 07, 08, 19 and 20) of propolis were deposited at the Laboratory of Self Medication, School of Pharmacy, Nihon University.

2.2. Chemicals

Chemicals were purchased as follows: DMBA from Sigma Chemical Co. (St. Louis, MO), TPA from Chemicals for Cancer Research, Inc. (Chicago, IL), and acetone from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Moronic acid was isolated from BP-AF-08 [10].

2.3. 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 (7 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed in an air-conditioned specific pathogen-free room (22–23°C) illuminated from 08:00–20:00. Food and water were available *ad libitum*.

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2.4. ASSAY OF TPA-INDUCED INFLAMMATION IN MICE

TPA (1 µg) dissolved in acetone (20 µl) was applied to the right ear only of mice by means of a micropipette. A volume of 10 µl was delivered to both the inner and outer surfaces of the ear. The samples or its vehicle, MeOH-CHCl₃ (1:1, 20 µl), as a control, were topically applied approximately 30 min before TPA treatment. Ear thickness was determined with a pocket thickness gauge having a range of 0–9 mm, graduated at 0.01-mm intervals and modified such that the contact surface area was increased to reduce loading when applied to the tip of the ear. Ear thickness was measured before treatment (*a*) and at 6 h after TPA treatment (*b* = TPA alone; *b'* = TPA plus sample). The following values were then calculated:

Edema A induced by TPA alone (*b* – *a*);

Edema B induced by TPA plus a sample (*b'* – *a*);

Inhibitory rate (%) = [(edema A – edema B)/edema A] × 100

Each value was the mean of individual determinations from 4 mice.

2.5. Two-Stage Mouse Skin Carcinogenesis Model

Each group of animals was composed of 15 mice housed five per cage. The back of each mouse was shaved with electric clippers, and mice were topically treated with DMBA (50 µg, 195 nM) in acetone (0.1 ml) for initiation treatment. One week after initiation, papilloma formation was promoted twice a week by application of TPA (1 µg, 1.7 nM) in acetone (100 µl) onto the skin. An ethanol extract of (1 mg) of BP-AF-08 or moronic acid (2 µmol) and their vehicle, acetone-dimethylsulfoxide (9:1, 100 µl) was applied topically 30 min before TPA treatment. Tumor incidence and number of papillomas were recorded weekly for 20 weeks.

2.6. Data Analysis

Statistical differences were verified by one-way analysis of variance followed by correction with Tukey-Kramer test (in Table 1). The ID₅₀ values and their 95% confidence intervals (95%CI) were obtained by nonlinear regression using GraphPad PRISM ver. 5.0 (intuitive Software for Science, San Diego, CA) (Table 2). Differences between experimental groups were compared by Mann-Whitney *U* exact test (in Figure 1A and Figure 2A) and Student's *t*-test (in Figure 1B and Figure 2B).

3. RESULTS

3.1. Inhibitory Effects of TPA-Induced Inflammatory Ear Edema in Mice

Various Brazilian propolis extracts were tested for their ability to reduce the intensity of TPA-induced ear edema (Table 1). This is the first report to find that ethanol extracts of Brazilian propolis inhibit tumor promoter-induced inflammation in mice. Moronic acid (Figure 1), a major component, had been isolated from BP-AF-08 [10]. Moronic acid inhibited TPA-induced inflammatory ear edema. As shown in Table 2, the ID₅₀ of moronic acid on TPA-induced inflammation is 379 nmol/ear. By comparison with standard drugs, moronic acid is a stronger inhibitor of TPA-induced inflammation than indomethacin.

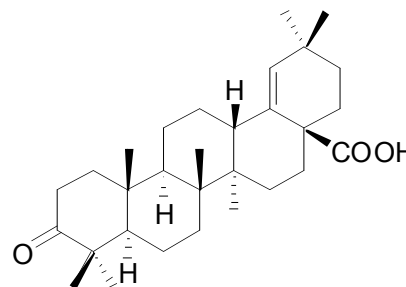


Figure 1: Chemical structure of moronic acid.

3.2. Inhibitory Effects of BP-AF-08 on Tumor Promotion

Ethanol extracts of BP-AF-08 inhibit tumor promotion by TPA after initiation with DMBA in mouse skin.

Ethanol extracts of BP-AF-08 moderately inhibited tumor promotion by TPA following initiation with DMBA. Figure 2A shows the time course of skin tumor formation in the group treated with DMBA plus TPA, with or without ethanol extracts of BP-AF-08. In the group treated with DMBA plus TPA, the first tumor appeared at week 5 and all 15 mice presented with tumors at week 12. In the groups treated with DMBA plus TPA and BP-AF-08, the first tumor appeared at week 7. The percentage of tumor-bearing mice treated with DMBA plus TPA was 100% at week 20, whereas that in the group treated with DMBA plus TPA and BP-AF-08 was 20%. Figure 2B shows the average number of tumors per mouse at week 20. The group treated with DMBA plus TPA produced 14.5 tumors per mouse, whereas the DMBA plus TPA and BP-AF-08 groups had 1.8 tumors per mouse. Thus, treatment with

Table 1: Features of Propolis, and the Inhibitory Effects of Ethanol Extracts on TPA-Induced Inflammation in Mice

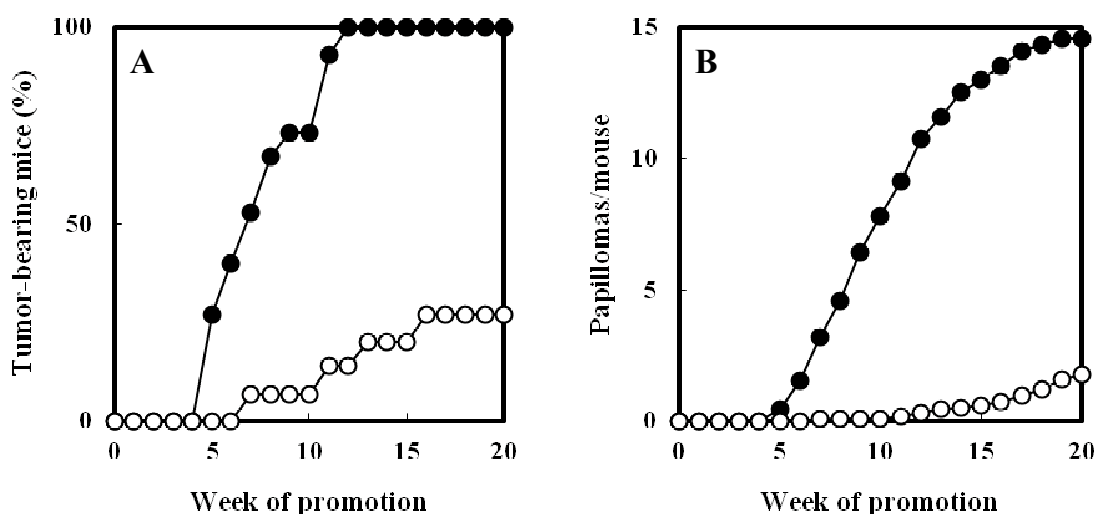
| Sample | Species ^a | Family ^a | State ^b | I.R. ^c (%) |
|--------|----------------------------------|---------------------|--------------------|-----------------------|
| AF-06 | <i>Baccharis erioclada</i> | Compositae | Rio Grande do Sul | 85* |
| AF-07 | <i>Baccharis dracunculifolia</i> | Compositae | Minas Gerais | 68* |
| AF-08 | <i>Myrceugenia euosma</i> | Myrtaceae | Rio Grande do Sul | 92* |
| AF-19 | <i>Baccharis caprarifolia</i> | Compositae | Parana | 76* |
| AF-20 | <i>Hyptis divaricata</i> | Labiatae | Bahia | 82* |

Note: ^aMajor botanical origins in areas where propolis was collected. ^bBrazilian states where propolis was collected. ^cInhibitory ratio at 1 mg/ear. * $p < 0.001$ by one-way ANOVA compared with the control group.

Table 2: Inhibitory Effects of Moronic Acid (Triterpene) from BP-AF-08 on TPA-Induced Inflammation in Mice

| Sample | ID ₅₀ ^a (nmol/ear) | 95% CI ^b (μmol/ear) |
|--------------|--|--------------------------------|
| Moronic acid | 379 | 318–452 |
| Indomethacin | 908 | 755–1,092 |

Note: ^aThe 50% inhibitory dose. ^b95% Confidence intervals.

**Figure 2:** Inhibitory effects of 70% ethanol extract of Brazilian propolis AF-08 on promotion of skin papillomas by TPA in DMBA-treated mice.

A: Percentage of mice bearing tumors; **B:** Average number of papillomas per mouse. ●, + TPA with vehicle; ○, TPA with 70% ethanol extract of Brazilian propolis AF-08 (1 mg/mouse). $n = 15$. Brazilian propolis AF-08-treated group was significantly different from the control group on Mann-Whitney U exact test ($P < 0.05$ = after week 6, 7; $P < 0.01$ = after weeks 8–20) and Student's t -test ($P < 0.05$ = after weeks 6–8; $P < 0.01$ = after weeks 9–20).

BP-AF-08 resulted in an 88% reduction in the average number of tumors per mouse at week 20.

3.3. Inhibitory Effects of Moronic Acid on Tumor Promotion

Moronic acid moderately inhibited tumor promotion by TPA following initiation with DMBA. Figure 3A shows the time course of skin tumor formation in the group treated with DMBA plus TPA, with or without moronic acid. In the group treated with DMBA plus

TPA, the first tumor appeared at week 5 and all 15 mice presented with tumors at week 13. In the groups treated with DMBA plus TPA and moronic acid, the first tumor appeared at week 10. The percentage of tumor-bearing mice treated with DMBA plus TPA was 100% at week 20, whereas that in the group treated with DMBA plus TPA and moronic acid was 20%. Figure 3B shows the average number of tumors per mouse at week 20. The group treated with DMBA plus TPA produced 9.3 tumors per mouse, whereas the DMBA plus TPA and moronic acid groups had 1.4 tumors per

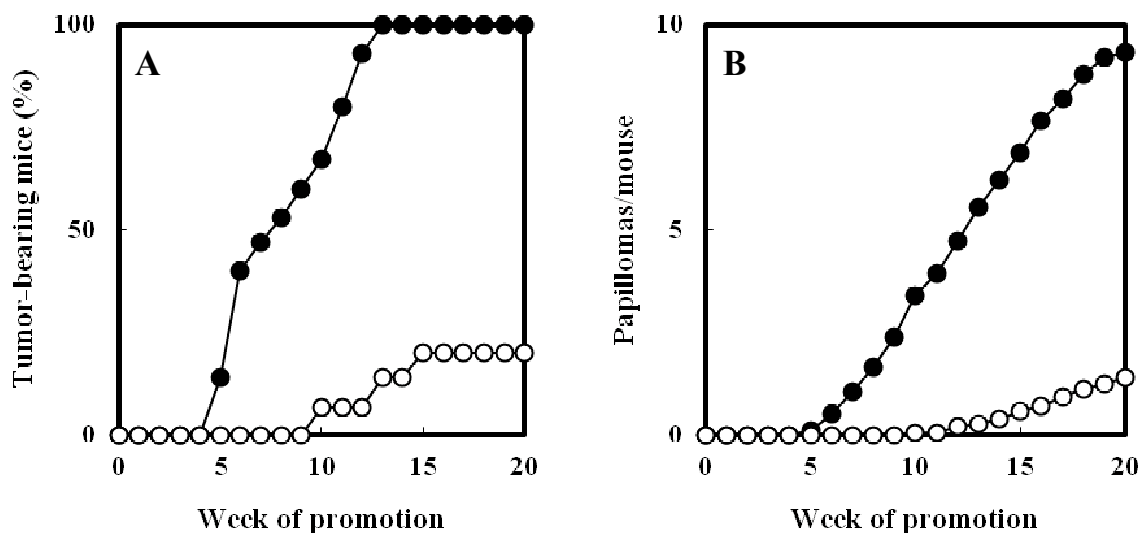


Figure 3: Inhibitory effects of moronic acid on promotion of skin papillomas by TPA in DMBA-treated mice.

A: Percentage of mice bearing tumors; **B:** Average number of papillomas per mouse. ●, + TPA with vehicle; ○, TPA with moronic acid (2 μ mol/mouse). $n = 15$. Moronic acid treated group was significantly different from the control group by Mann-Whitney U exact test ($P < 0.05 =$ after week 6; $P < 0.01 =$ after week 7–20) and Student's t test ($P < 0.05 =$ after weeks 6–17; $P < 0.01 =$ after weeks 18–20).

mouse. Thus, treatment with moronic acid resulted in an 85% reduction in the average number of tumors per mouse at week 20.

4. DISCUSSION

Propolis is a resinous hive product collected by honeybees from various plant sources. It has a long history of use in folk medicine dating back to at least 300 BC [11], and it has been reported to possess various biological activities, including anti-cancer, antioxidant, anti-inflammatory, anti-bacterial, anti-fungal and anti-hepatotoxic actions [11–13].

Tropical propolis has been reported to have different chemical compositions and pharmacological activities from that produced in temperate zones [6,14,15]. Even among Brazilian propolis types, the chemical composition depends on the vegetation in the area where it is harvested.

Five different propolis extracts inhibited TPA-induced inflammatory ear edema. BP-AF-08 suppressed tumor promotion by TPA in two-stage carcinogenesis in mouse skin. A major component of BP-AF-08, moronic acid, markedly inhibited TPA-induced inflammatory ear edema in mice. 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 in a mouse skin model [16,17]. Many triterpenoids have been found to inhibit tumor promotion in two-stage carcinogenesis in

mouse skin [17,18]. In other words, moronic acid appears to inhibit tumor promotion in two-stage carcinogenesis in mouse skin. As expected, moronic acid markedly inhibited tumor promotion by TPA following initiation with DMBA in mouse skin. BP-AF-08 may contribute to the prevention of cancer.

BP-AF-08 has also been shown to exhibit a variety of biological activities, including anti-HIV [10], anti-influenza virus [19] and anti-herpes virus [20] actions. It has also been reported that moronic acid, an important component of this propolis, shows anti-viral [21–25] and cytotoxic [26] activities. Furthermore, we are going to isolate the active components from BP-AF-08. In addition, the bioactive elucidation of BP-AF-08 in the molecular level is necessary.

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

FINANCIAL DISCLOSURE

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ABBREVIATIONS

BP-AF-08 = Brazilian propolis AF-08

DMBA = 7,12-Dimethylbenz[a]anthracene

HIV = Human immunodeficiency virus

TPA = 12-O-Tetradecanoylphorbol-13-acetate

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