Inhibitory Effects of Chaga (*Inonotus Obliquus*) on Tumor Promotion in Two-Stage Mouse Skin Carcinogenesis

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Abstract: The methanol extract of chaga (sclerotia of *Inonotus obliquus*) inhibited the promoting effect of 12-*O*-tetradecanoylphorbol-13-acatate (TPA) applied twice weekly on skin tumor formation in mice initiated with 7,12-dimethylbenz [*a*] anthracene. The methanol extract from chaga led to the isolation of eight triterpenoids (**1**-**8**). The anti-inflammatory activity of the isolated lanostane-type triterpenes was evaluated against TPA-induced inflammatory ear edema in mice. These compounds showed markedly anti-inflammatory effects, with a 50% inhibitory dose of 125-458 nmol/ear.

Keywords: Chaga, *Inonotus obliquus*, lanostane-type triterpene, antitumor promotion, anti-inflammation, two-stage carcinogenesis.

1. INTRODUCTION

Our studies have illustrated that components from mushrooms inhibit tumor promotion in mouse skin two-stage carcinogenesis [1-6]. In addition, extracts from mushrooms inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory ear edema. Active components were thus further isolated from *Hypsizigus marmoreus* [1], *Poria cocos* [3], *Ganoderma lucidum* [5] and *Polyporus umbelletus* [6].

Since the sixteenth century, the sclerotia of *Inonotus obliquus* (Hymenochaetaceae), chaga, have been used as a folk medicine for cancer [7]. Recently, it has been reported that chaga contains triterpenoids [8-26] and phenolics [27-29]. On the other hand, we found that lanostane-type triterpenoids inhibit tumor promotion in mouse skin two-stage carcinogenesis [4,5].

Here, we found that methanol extract of chaga (MEC) showed an inhibitory effect on tumor promotion by TPA following initiation with 7,12-dimethylbenz [*a*] anthracene (DMBA) in mice. In addition, eight triterpenoids were subsequently isolated from MEC.

2. MATERIALS AND METHODS

2.1. General

Optical rotations were measured with a JASCO P-1020 polarimeter. IR spectra were recorded as KBr pellets on a JASCO FT/IR-300E spectrometer. UV spectra were measured on a JASCO V-550 spectrophotometer in absolute $CHCl_3$. HR-EI-MS, EI-MS and FAB-MS were measured with a JEOL JMS-GCMATE mass spectrometer at an ionization voltage of 70 eV. CD spectra were recorded in $CHCl_3$ on a JASCO J-600 spectrometer. ^1H and ^{13}C NMR spectra were obtained on a JEOL JNM-LA500 (^1H, 500 MHz; ^{13}C, 125 MHz) spectrometer. $CDCl_3$ was used as the solvent and TMS as the internal standard. Column chromatography was carried out with Sephadex LH-20 (18-111 μm; 30 × 1,000 mm; Amersham Biosciences) and Silica gel 60 (70-230 mesh; 45 × 220 mm; Merck). HPLC (Inertsil ODS-EP, 10 × 250 mm; GL Science Inc.) was run on a JASCO PU-2089 Plus instrument equipped with a JASCO MD-2015 Plus detector and JASCO CO-2060 thermostat.

2.2. Chemicals

TPA was purchased from Chemicals for Cancer Research, Inc. (Eden Prairie, MN). DMBA, indomethacin and hydrocortisone were obtained from Sigma Chemical Co. (St. Louis, MO). Acetone, chloroform and methanol were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

2.3. Material and Extraction

Chaga, the sclerotia of *Inonotus obliquus* (Pers.: Fr.) Pil., was obtained from Kinokuniya Kan Yakkyoku Co. (Tokyo, Japan) in 2006. The sample was identified by one of the authors (Yasukawa). The voucher specimen (SM-0603) was deposited in the laboratory of Self Medication at School of Pharmacy, Nihon University (Chiba, Japan). The crushed dry chaga, the sclerotia of *I. obliquus* (1.0 kg), was extracted three

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times for 3 days each with MeOH (5 L) at room temperature to given an extract (32.4 g). This extract (30 g) was partitioned between EtOAc-H_2O (2.5:2.5 L). EtOAc extract (20.6 g) was partitioned between *n*-haxane (1.9 L) and MeOH-H_2O (1.9:0.2 L) to obtain *n*-hexane (9.8 g) and MeOH-H_2O (10.0 g) extracts. The H_2O layer was partitioned between with *n*-BuOH (2.5 L) to given *n*-BuOH extract (5.5 g) and H_2O extract (6.3 g).

2.4. Isolation and Identification

The MeOH-H_2O fraction (10.0 g) was subjected to column chromatography (CC) on Sephadex LH-20 column chromatography (18-111 μm; 30 × 1,000 mm), using MeOH-$CHCl_3$ (1:1) to obtain four fractions, fraction 1 (0.65 g; 1-100 mL), 2 (5.2 g; 100-2600 mL), 3 (1.62 g; 2600-3700 mL), 4(1.74 g; 3700-6000 mL).

The inhibitory fraction 2 (5 g) was further subjected to CC (silica gel 60, 70-230 mesh; 4.5 cm × 220 mm) using *n*-hexane/EtOAc (4:1, 1.5 mL/min) to give fraction 2-1 (34.8 mg; 1-130 mL), fraction 2-2 (32.6 mg; 130-1150 mL), fraction 2-3 (64.7 mg; 1150-2300 mL). Elution was continued with *n*-hexane/EtOAc 1:1 to give residues fraction 2-4 (757.5 mg; 1-1140 mL), fraction 2-5(522.1 mg; 1140-2500 mL) and subsequent column chromatography with *n*-hexane/EtOAc (1:3, 1.5 mL/min) to give residues fraction 2-6(1.1 g; 1-1970 mL), fraction 2-7(787.6 mg; 1970-6000 mL), respectively.

The active fraction 2-2 was then purified by HPLC (ODS, 85% MeOH, 4.0 mL/min) to give compound **3** (5.2 mg, t_R 29 min). Fraction 2-3 was subjected to HPLC (ODS, 82% MeOH, 4.0 mL/min) to give compound **5** (3.4 mg, t_R 68 min). Fraction 2-4 was purified with HPLC (ODS, 74% MeOH, 4.0 mL/min) to give compounds **4**(242.9 mg, t_R 72 min), **5**(18.0 mg, t_R 85 min), **7**(17.2 mg, t_R 38 min) and **8**(32.0 mg, t_R 53 min). Fraction 2-5 was separated with HPLC (ODS, 67% MeOH, 4.0 mL/min) to give compounds **1**(10.5 mg, t_R 36 min), **2**(8.3 mg, t_R 155 min), **4**(118.6 mg, t_R 164 min) and **6**(10.6 mg, t_R 180 min).

Compounds **1**-**8**, the lanostane derivatives methoxyinonotsutriol (**1**), inotolacton B (**2**), lanosterol (**3**), inotodiol (**4**), 3β-hydroxylanosta-8, 24-dien-21-al (**5**), uvariol (**6**), and 3β, 22-dihydroxylanosta-7, 9(11), 24-triene (**7**), and the lupane derivative betulin (**8**), were elucidated by comparison of spectral data with those in the literature [22-25, 30-32].

2.5. Animals

The experiment with mice was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Nihon University. Specific pathogen free (SPF) female ICR mice were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals, four per polycarbonate cage, were housed in an air-conditioned SPF room at 24 ± 2 °C, 12 hour light/dark cycle. Food and water were available *ad libitum*.

2.6. TPA-Induced Inflammation Assay in Mice

For the protocol for this *in vivo* assay [1-3].

2.7. Two-Stage Carcinogenesis Experiment

The back of mice(7 weeks old) were shaved with electric clippers. Initiation was accomplished by a single topical application of 50 μg DMBA. Promotion with 1.0 μg TPA, applied twice weekly, was begun 1 week after the initiation. MEC (1.0 mg) or its vehicle, acetone-water-dimethylsulfoxide (8:1:1, 100 μL), was applied topically 30 min before each TPA treatment. DMBA and TPA were dissolved in acetone, and applied to the shaved area in a volume 100 μL using a micropipette. The number and diameter of a skin tumors were measured every other week, and the experiment was continued for 20 weeks. Experimental and appropriate control groups each consisted of 15 mice.

2.8. 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.

3. RESULTS AND DISCUSSION

Anti-inflammatory activity may play an important role in the mechanism of antitumor promotion, and several anti-inflammatory substances are known to inhibit the action of tumor promoters. The inhibitory effects of TPA-induced inflammation have been shown to roughly parallel their inhibitory activities against TPA-induced tumor promotion [33]. MEC inhibited TPA-induced inflammation in mice, as shown in Table **1**. This suggests that MEC possessed inhibitory effects on inflammation as indicated by the suppression of tumor

Table 1: Inhibitory Effect of Chaga on TPA-Induced Inflammatory Ear Edema

Sample	IR
MeOH extract (1 mg/ear)	84**
EtOAc layer (1 mg/ear) of MeOH extract	92**
n-Hexane layer (1 mg/ear) of MeOH extract	89**
MeOH-H_2O layer (1 mg/ear) of MeOH extract	94**
n-BuOH layer (1 mg/ear) of MeOH extract	21
H_2O layer (1 mg/ear) of MeOH extract	10
Fraction 1 (0.5 mg/ear) from MeOH-H_2O layer of MeOH extract	80**
Fraction 2 (0.5 mg/ear) from MeOH-H_2O layer of MeOH extract	87**
Fraction 3 (0.5 mg/ear) from MeOH-H_2O layer of MeOH extract	27
Fraction 4 (0.5 mg/ear) from MeOH-H_2O layer of MeOH extract	33*

Note: IR: Inhibitory ratio at 1 mg/ear. * $P < 0.05$; ** $P < 0.01$ by one-way ANOVA compared with the control group.

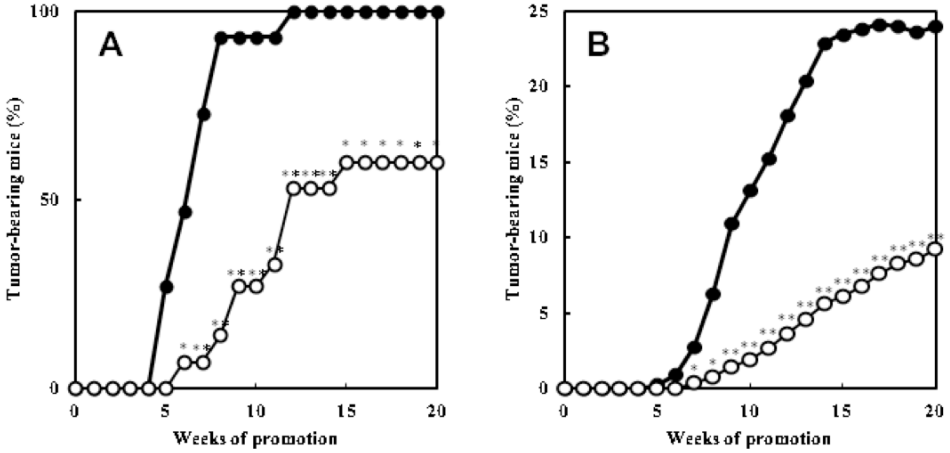

Figure 1: Inhibitory effect of MeOH extract of chaga on tumor promotion of skin papillomas by TPA in DMBA-initiated mice.

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 the MeOH 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 MeOH extract of chaga. 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 ** $P < 0.01$.

promotion in mouse skin. We further determined the effects of MEC on chronic inflammatory diseases using a two-stage carcinogenesis in mouse skin.

The activities, evaluated by both the rate (%) of pappilloma-bearing mice and the average number of papillomas per mouse were compared with those of a positive control. As shown in Figure **1**, on the positive control, 100% of mice bore pappilomas at week 12 of promotion, and 23.7 pappilomas were formed per mouse after 20 weeks of promotion. When EMC were applied before TPA treatment, the delayed the formation of pappilomas as follows. In the group treated with EMC, only 60% of the mice bore pappilomas, even at 20 weeks of promotion (Figure **1A**). Also, EMC reduced the number of pappilomas per mouse as follows. In the case of EMC, 9.3 pappillomas were formed per mouse after 20 weeks of promotion. EMC exhibited 61% inhibition at week 20, as shown Figure **1B**. There were no differences regarding body weight between the control and treated group during experiment (data not shown).

MEC was purified by column chromatography on Sephadex LH-20 and silica gel followed by preparative reverse phase HPLC, yielding seven known lanostane derivatives, methoxyinonotsutriol (**1**), inotolacton B (**2**), lanosterol (**3**), inotodiol (**4**), 3β-hydroxylanosta-8, 24-dien-21-al (**5**), uvariol (**6**) and 3β, 22-dihydroxylanosta-7, 9(11), 24-triene (**7**), and the lupane derivative betulin

(**8**). Active components were then isolated from MEC (Figure **2**). The structures of these compounds were elucidated by their spectroscopic data with those reported in the literature. These compounds showed inhibitory activity against TPA-induced ear edema. The ID_{50} values for **1**-**8** on TPA-induced inflammation were between 125-458 nmol/ear, as shown in Table **2**. In comparison with standard drugs, these triterpenoids had higher activity than indomethacin (ID_{50}: 908 nmol/ear), 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 TPA, a well-known tumor promoter, in a mouse skin model [33]. In our study, we found that topical application of MEC postponed the period of 50% of papilloma bearers and all isolated compounds from MEC exhibited potent anti-inflammatory effects on *in vivo* assay. Chaga triterpenes may be found to inhibit inflammation and tumor promotion in mouse skin. In addition, the triterpenes of chaga showed intermediate inhibitory effects when compared with the triterpenes of *Poria cocos* [3] and *Ganoderma lucidum* [5]. Lanostane-type triterpenes from *Poria cocos* inhibited pospholipase A_2 [34], which is related to inflammation, and these lanostane-type triterpenoids (**1**-**7**) may suppress the same enzyme. Many triterpenes, widely distributed in edible mushrooms and plants, are now known to inhibit the tumor promoting activities of TPA in mice and this suggests that they may be important

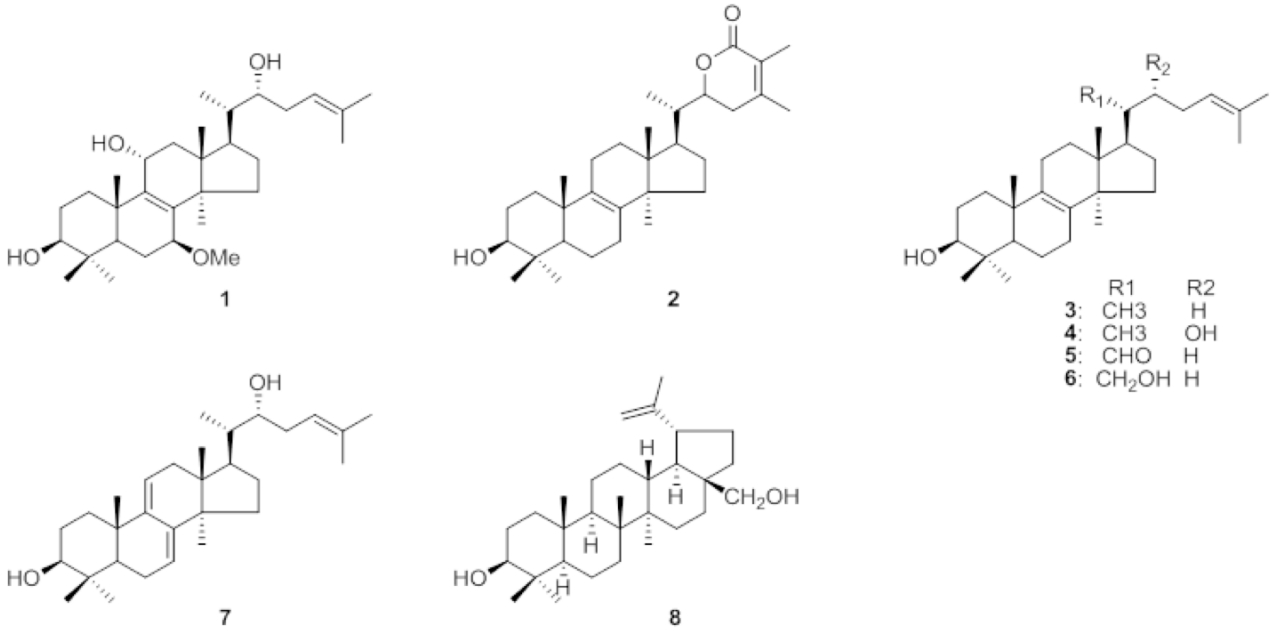

Figure 2: Chemical structures of compounds **1**-**8** from chaga.

Table 2: Inhibitory Effect of Triterpenoids from Chaga on TPA-Induced Inflammatory Ear Edema

Compound	ID50 (nmol/ear)	95% CI (nmol/ear)
Methoxyinonotsutriol (1)	272	223-288
Inotolacton B (2)	265	227-353
Lanosterol (3)	458	416-503
Inotodiol (4)	125	103-152
3β-Hydroxylanosta-8, 24-dien-21-al (5)	389	310-448
Uvariol (6)	134	98-184
3β,22-Dihydroxylanosta-7,9(11),24-triene (7)	335	256-439
Betulin (8)	448	408-495
Indomethacina	908	755-1092

Note: [a] Reference compounds. ID50: 50% Inhibitory dose. 95% CI: 95% Confidence intervals.

dietary additives for the chemoprevention of cancer. This is the first report to find that MEC inhibits TPA-induced inflammatory ear edema in mice. Furthermore, MEC inhibits tumor promotion by TPA following initiation with DMBA in mice. In addition, the bioactive elucidation of chaga at the molecular level is necessary.

CONFLICTS OF INTEREST

There are no potential conflicts of interest to disclose.

FINANCIAL DISCLOSURE

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ABBREVIATIONS

95% CI = 95% Confidence intervals

DMBA = 7, 12-Dimethylbenz[a] anthracene

EMC = Methanol extract of chaga

ID_{50} = 50% Inhibitory dose

MeOH = Methanol

TPA = 12-*O* -Tetradecanoylphorbol-13-acetate

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