Inhibitory Effects of the Aerial Parts of *Epimedium koreanum* on TPA-Induced Inflammation and Tumour Promotion in Two-Stage Carcinogenesis in Mouse Skin

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Abstract: Cancer prevention by supplements offers the most cost-effective long-term health strategy. Methanol extracts from the aerial parts of *Epimedium koreanum* were previously found to inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory ear oedema and tumour promotion by TPA in the two-stage mouse skin carcinogenesis model. Four prenyl flavonol glycosides (**1**–**4**) were isolated from the active fraction of this extract, and were identified. The isolated compounds showed inhibitory activity against TPA-induced ear inflammatory ear oedema. The 50% inhibitory dose (ID_{50}) of icariin (**1**), epimedin A (**2**), epimedin B (**3**) and epimedin C (**4**) for TPA-induced inflammation ranged from 114 to 255 nmol/ear, suggesting greater potency than indomethacin (ID_{50}: 908 nmol/ear), an anti-inflammatory drug. Thus, the epimedium herb may be useful in cancer prevention.

Keywords: Cancer chemoprevention, antitumour-promoting activity, two-stage carcinogenesis, prenyl flavonol glycosides, *Epimedium koreanum.*

1. INTRODUCTION

The prevention of cancer is an urgent priority in the field of public health. Animal models have demonstrated experimentally that chronic inflammation can lead to the development of various forms of cancer, while providing further insights into possible mechanisms. Skin tumour are induced by administration of carcinogens such as 7,12-dimethylbenz[*a*]anthracene (DMBA), followed by repeated administration of tumour promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [1,2]. Our previous studies have confirmed that constituents from natural sources, such as medicinal plants and fungi, inhibit tumour promotion by TPA in two-stage carcinogenesis in mouse skin [3-6]. Many tumour promoters have inflammatory activity [7], and based on our previous experience, we therefore focused on natural sources to screen for novel inhibitors as preventive agents.

Epimedium herb (Berberidaceae) is one of the important medicinal herbs recorded in the Chinese, Korean and Japanese Pharmacopoeias [8-10]. This medicinal herb has several base source plants; *E*. *pubescens* Maxim., *E*. *brevicornu* Maxim., *E*. *wushanense* T. S. Ying, *E*. *sagittatum* Maxim., *E*. *koreanum* Nakai, *E*. *grandiflorum* Morren var. *thunbergianum* Nakai, and *E*. *sempervirens* Nakai. *Epimedium sagittatum* Maxim. was first described as a medicinal herb in the Shen Nong Canon of Herb (written AD 25-220). This herb acts mainly as an aphrodisiac and as a tonic for the liver and kidneys [11]. It dilates blood vessels, lowers blood pressure, and can be taken internally for asthma, bronchitis, colds or numb extremities, arthritis, lumbago, impotence, involuntary and premature ejaculation, high blood pressure and absent-mindedness [11]. In chemical studies of *E. koreanum* to date, the isolation and structural determination of prenyl flavonoids, sterols, lignin, phenol glycosides, phenylethanoid glycosides and sesquiterpenoids have been reported [12].

In the present study, methanol extracts from the aerial parts of *E*. *koreanum* were found to inhibit TPA-induced tumour promotion during two-stage carcinogenesis in mouse skin. Four prenyl flavonol glycosides (**1**–**4**) were subsequently isolated from the methanol extracts of aerial parts of *E*. *koreanum*. The 50% inhibitory doses (ID_{50}) of these compounds for TPA-induced inflammatory ear oedema ranged from 114 to 252 nmol/ear, which suggested greater potency than indomethacin (ID_{50}: 908 nmol/ear).

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2. MATERIAL AND METHODS

2.1. Analytical Methods

High-resolution electron impact mass spectrometry and electron impact mass spectrometry were performed using a JEOL JMS-GC MATE mass spectrometer at an ionisation voltage of 70 eV. ^{1}H- and ^{13}C-nuclear magnetic resonance spectra were obtained on a JEOL JNM-LA500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer. Dimethyl sulfoxide (DMSO) was used as the solvent, and tetramethyl silane was used as the internal standard.

2.2. Chemicals

7,12-Dimethylbenz[a]anthracene (DMBA), indomethacin, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TPA was obtained from Chemical Cancer Research, Inc. (Minnesota, MN, USA). Methanol, chloroform, ethyl acetate, *n*-butyl alcohol, acetone and *n*-hexane were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan)

2.3. Plant Material

E. koreanum Nakai was obtained from a local market in Kyungdongitiba, Seoul, in March 2006, and was identified by Professor W.-K. Whang of Chung-Aung University (Seoul, Korea). Voucher specimens “SM0604” were deposited at the Laboratory of Self Medication, School of Pharmacy, Nihon University.

2.4. Extraction Procedur

Dried aerial parts of *E. koreanum* were extracted with methanol at room temperature, and the solvent was evaporated *in vacuo*. Each extract was examined for inhibitory activity against TPA-induced ear oedema and tumour promotion in mice.

2.5. Isolation

The aerial parts of *E. koreanum* (1 kg) was extracted five times for 3 days with methanol at room temperature to give an extract (46.8 g). This extract (45 g) was partitioned between ethyl acetate-water (1:1) to yield an ethyl acetate extract (18.7 g). The ethyl acetate extract (18.0 g) was partitioned between *n*-hexane-methanol-water (19:19:2), which afforded *n*-hexane extract (2.83 g) and methanol-water extract (15.1 g), respectively. The water solution was partitioned between *n*-butyl alcohol-water (1:1), yielding an *n*-butyl alcohol extract (6.19 g) and a water extract (19.8 g), respectively.

The methanol-water extract (5.0 g) was subjected to column chromatography (CC) on Sephadex LH-20 (18 – 111 μm, 30 × 1,000 mm; Amersham Biosciences, Uppsala, Sweden) using methanol to obtain five fractions: Fr-MW1 (0.98 g); Fr-MW2 (0.92 g); Fr-MW3 (1.14 g); Fr-MW4 (1.01 g); and Fr-MW5 (0.85 g). Fr-MW3 (1.0 g) was subjected to CC on Sephadex LH-20 using 95% and 90% methanol to yield compound **1** (156 mg). The *n*-butyl alcohol extract (5.0 g) was subjected to CC on Sephadex LH-20 using 90% methanol to obtain six fractions: Fr-B1 (0.36 mg); Fr-B2 (0.87 mg); Fr-B3 (1.26 g); Fr-B4 (0.85 mg); Fr-B5 (0.76 mg); and Fr-B6 (0.85 mg). Fr-B3 (1.2 g) was further separated on Sephadex LH-20 using 85% methanol to obtain eight fractions: Fr-B3-1 (198 mg); Fr-B3-2 (215 mg); Fr-B3-3 (519 mg); Fr-B3-4 (108 mg); and Fr-B3-5 (165 mg). Fr-B3-3 (510 mg) was then purified by repeated reversed-phase preparative HPLC (RP-C18, methanol/water, 70:30) to afford **2** (9.0 mg), **3** (10 mg) and **4** (7.0 mg), respectively.

2.6. Identification

Identification of prenyl flavone glycosides **1** (icariin) [13], **2** (epimedin A), **3** (epimedin B), and **4** (epimedin C) [14] was performed by spectral comparison with literature data.

2.7. 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 were obtained from Japan SLC Inc., Shizuoka, Japan. The animals were housed in an air-conditioned, specific-pathogen-free room (24 ± 2°C) lit from 08:00 to 20:00. Food and water were available *ad libitum*.

2.8. TPA-Induced Inflammation

TPA (1 μg) dissolved in acetone (20 μL) was applied to the right ear of the ICR mice using a micropipette. The same volume (10 μL) was applied to both inner and outer surfaces of the ear. Test samples or their vehicles [chloroform–methanol (1:1) or chloroform–methanol–water (2:1:1)] used as controls, were topically applied about 30 min before the 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 to increase the contact surface area to reduce tension, was applied to the tip of the ear. Ear thickness was determined before the TPA

treatment (a), and oedema was measured at 6 h after the TPA treatment (b: TPA with vehicle; b': TPA with sample).

The following values were then calculated:

Oedema A = oedema induced by TPA with vehicle (b – a);

Oedema B = oedema induced by TPA with sample (b' – a);

Inhibition ratio (%) = [(oedema A – oedema B)/oedema A] × 100.

Each value was calculated as the mean of individual determinations from four mice.

2.9. Two-Stage Carcinogenesis Experiments

The backs of the mice (age, 7 weeks) were shaved using electric clippers once a week to remove hair. DMBA and TPA were dissolved in acetone and applied to the shaved area in a volume of 100 µL using a micropipette. The initiation was accomplished by a single topical application of 50 µg of DMBA. Promotion using 1 µg of TPA, applied two times a week, was started one week after the initiation. The methanol extract of the aerial parts of *E. koreanum* (1.0 mg/mouse) or its vehicle, acetone–DMSO–water (8:1:1, 100 µL), was topically applied 30 min before each TPA treatment. The number and diameter of skin tumours were determined every week, and the experiment was continued for 20 weeks. The experimental and control groups consisted of 15 mice each.

2.10. Statistical Analysis

The 50% inhibitory dose (ID_{50}) values and 95% confidence intervals (95% CI) were calculated by nonlinear regression using GraphPad Prism v. 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between experimental groups were compared using Student's *t*-test and Mann–Whitney *U* exact test.

3. RESULTS AND DISCUSSION

As can be seen in Table **1**, extracts from aerial parts of *E. koreanum* inhibited TPA-induced inflammation in mice. The inhibitory effects of the methanol extracts from aerial parts of *E. koreanum* in a two-stage carcinogenesis test on mouse skin using DMBA as an initiator and TPA as a tumour promoter were then investigated. Figure **1A** illustrates the time course of skin tumour formation in the groups treated with DMBA plus TPA, with or without the methanol extract from aerial parts of *E. koreanum*. The first tumour appeared at week 5 in the group treated with DMBA plus TPA. In the group treated with DMBA plus TPA and methanol

Table 1: Inhibitory Effects of Extracts and Fractions from the Aerial Parts of *E. koreanam* on TPA-Induced Inflammatory Ear Oedema

Sample	I.R.
Methanol extract (1 mg/ear)	66**
Ethyl acetate extract (1 mg/ear) from methanol extract	61**
n-Butyl alcohol extract (1 mg/ear) from methanol extract	82**
Water extract (1 mg/ear) from methanol extract	18
Fr-MW1 (0.5 mg/ear) from methanol-water extract of ethyl acetate extract	13
Fr-MW2 (0.5 mg/ear) from methanol-water extract of ethyl acetate extract	45**
Fr-MW3 (0.5 mg/ear) from methanol-water extract of ethyl acetate extract	85**
Fr-MW4 (0.5 mg/ear) from methanol-water extract of ethyl acetate extract	30*
Fr-MW5 (0.5 mg/ear) from methanol-water extract of ethyl acetate extract	6
Fr-B1 (0.5 mg/ear) from *n*-butyl alcohol extract	11
Fr-B2 (0.5 mg/ear) from *n*-butyl alcohol extract	38**
Fr-B3 (0.5 mg/ear) from *n*-butyl alcohol extract	78**
Fr-B4 (0.5 mg/ear) from *n*-butyl alcohol extract	28*
Fr-B5 (0.5 mg/ear) from *n*-butyl alcohol extract	10
Fr-B6 (0.5 mg/ear) from *n*-butyl alcohol extract	5

I.R.: Inhibitory ratio at 0.5 or 1.0 mg/ear. *$p < 0.05$, **$p < 0.01$.

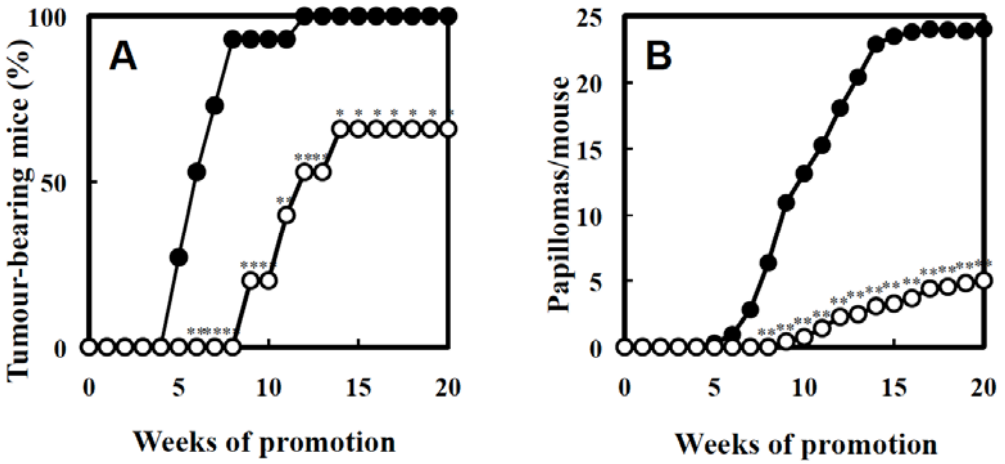
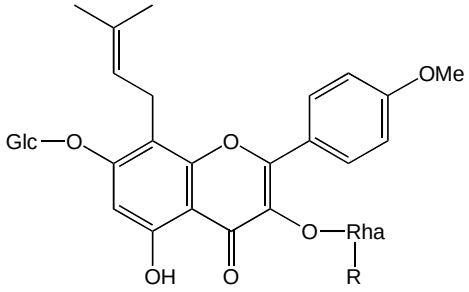


Figure 1: Inhibitory effects of methanol extracts from aerial parts of *E*. *koreanum* on tumour promotion of skin papillomas by TPA in DMBA-initiated mice. From 1 week after initiation with a single topical application of 50 μg of DMBA, 1 μg of TPA was applied twice weekly. Topical application of methanol extract (1 mg) and vehicle was performed 30 min before each TPA treatment. Data are expressed as a 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 aerial parts of *E*. *koreanum*. Treated group was determined to be statistically different from control group by Mann-Whitney *U* exact test (**A**), and by Student's *t*-test (**B**). * $p<0.05$, ** $p<0.01$.

extract from aerial parts of *E*. *koreanum*, the first tumour appeared at week 9. The percentage of tumour-bearing mice treated with DMBA plus TPA was 100% at week 12, whereas that in the group treated with DMBA plus TPA and methanol extract from aerial parts of *E*. *koreanum* was 66%. Figure **1B** shows the average number of tumours per mouse. The group treated with DMBA plus TPA produced 24.0 tumours per mouse at week 20; the group treated with DMBA plus TPA and methanol extract from aerial parts of *E*. *koreanum* had 5.0 tumours per mouse. Treatment with methanol extract from aerial parts of *E*. *koreanum* caused a 79% reduction in the average number of tumours per mouse at week 20.

Active components were then isolated from the methanol extract from aerial parts of *E. koreanum* (Figure **2**). Isolated compounds showed inhibitory activity against TPA-induced ear inflammatory oedema. As can be seen in Table **2**, the ID_{50} values of **1** – **4** on TPA-induced inflammation were between 114 – 252 nmol/ear, respectively. In comparison with standard drugs, these components (**1**–**4**) had greater activity than indomethacin (ID_{50} = 908 nmol/ear), an anti-inflammatory drug.

The proinflammatory cytokine tumour necrosis factor (TNF)-α is the most important mediator of inflammation and is a well-known endogenous tumour promoter, as previous findings have shown that mice deficient in TNF-α have fewer skin tumours after DMBA and TPA application [15]. Xu *et al*. reported that pretreatment with icariin (**1**) attenuated acute lung inflammation by inhibiting mRNA expression of TNF-α, interleukin-6, metalloproteinase cyclooxygenase-2, and inducible nitric oxide synthase in the lungs of lipopolysaccharide-treated mice. In addition, icariin (**1**) suppressed the secretion of TNF-α, prostaglandin-E_2 and nitric oxide, as well as nuclear factor-κB p65 activation [16].



	R
Icariin (**1**)	H
Epimedin A (**2**)	Glc
Epimedin B (**3**)	Xyl
Epimedin C (**4**)	Rha

Figure 2: Chemical structures of flavonoids from aerial parts of *Epimedium koreanum.*

This is the first report to confirm that methanol extracts of aerial parts of *E*. *koreanum* inhibit tumour promoter-induced inflammation in mice. Furthermore, this methanol extract inhibits tumour promotion by TPA following initiation with DMBA on ICR mouse skin. In addition, four active components, icariin (**1**) and epimedin A (**2**), B (**3**) and C (**4**), were isolated from the active fractions of the methanol extracts of aerial parts of *E*. *koreanum*.

Table 2: Inhibitory Effects of Components from *E. koreanam* on TPA-Induced Inflammatory Ear Oedema

Components and standard drugs	ID_{50}	95% CI
	(nmol/ear)	
Icariin (**1**)	122	69.1 ~ 215
Epimedin A (**2**)	252	145 ~ 438
Epimedin B (**3**)	114	82.2 ~ 158
Epimedin C (**4**)	192	133 ~ 279
Hydrocortisone	69.1	64.3 ~ 75.4
Indomethacin	908	755 ~ 1.092

ID_{50}: 50% inhibitory dose. 95% CI: 95% confidence intervals.

CONFLICT OF INTERESTS

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

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