【Short Communication】**Inhibitory Effect of Eucommia Bark on Tumour Formation in a Mouse Model of Two-Stage Skin Carcinogenesis**

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[ABSTRACT]

Cancer prevention is an important issue in the field of public health. In Oriental countries, *Eucommia* bark (the bark of *Eucommia ulmoides*) is used in tonics and anti-hypertensive medicines. *Eucommia* bark has inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. We demonstrated that at a dose of 1 mg/mouse, a methanol extract of *Eucommia* bark markedly inhibited the tumour-promoting activity of TPA in mice with skin tumour formation following initiation with 7,12-dimethylbenz[*a*]anthracene (DMBA). These results suggest the potential use of *Eucommia* bark in cancer prevention.

[Key words]

Eucommia bark, cancer prevention, two-stage carcinogenesis

INTRODUCTION

The chemoprevention of cancer is an urgent priority in the field of public health. It is important that the normal daily diet promotes the prevention of cancer. Our previous studies have illustrated that constituents of herbal medicines can inhibit tumour promotion in a mouse model of two-stage skin carcinogenesis¹⁾.

In Japan, the leaves of *Eucommia ulmoides* have been used in tea for the regulation of blood pressure, and the bark of *E. ulmoides* is incorporated in herbal medicines used as tonics and anti-hypertensive agents²⁾. The chemical constituents of *E. ulmoides* reportedly include lignins and iridoids^{3–8)}. In this paper, we demonstrated that the topical application of a methanol extract from *Eucommia* bark successfully inhibited tumour promotion in a mouse model of two-stage skin carcinogenesis, which was initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In our study, methanol extract was suitable for extraction of the active components than water extract⁹⁾. The reason that used methanol is because we expected active component to a low molecular compound. The water extract is suitable for the extraction of the high molecular compound, but is not suitable for the extraction of the low molecular compound.

MATERIALS AND METHODS**1 Materials**

Dried barks and dried leaves of *E. ulmoides* Oliver were sourced from Kinokuniya Kan-Yaku Kyoku Co., Tokyo, Japan. Voucher specimens “SM0204” and “SM0205” were deposited at the School of Pharmacy, Nihon University.

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2 Chemicals

TPA was sourced from Chemicals for Cancer Research Inc., Minnesota, Mo, USA. DMBA, dimethylsulfoxide and indomethacin were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Acetone, chloroform and methanol were obtained from Tokyo Kasei Kogyo Co., Ltd., Japan.

3 Extraction Procedure

Dried barks (100 g) and dried leaves (100 g) of *E. ulmoides* were extracted three times for 3 days with methanol (1,000 ml) at room temperature. The solvents were evaporated in a vacuum to dryness and the extracts were obtained bark extract (dark brown, yield: 7.8 g) and leaf extract (dark green, yield: 8.5 g), respectively. Each extract was examined for inhibitory activity against TPA-induced ear oedema and TPA-induced tumour promotion.

4 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 (SLC Inc., Shizuoka, Japan) were housed in an air-conditioned, specific pathogen-free room ($24 \pm 2^\circ\text{C}$) that was lit from 08:00 h to 20:00 h. Food and water were provided *ad libitum*.

5 Assay of TPA-induced inflammation in mice

TPA (1 μg) dissolved in acetone (20 μL) was applied to the right ears of the ICR mice using a micropipette. A volume of 10 μL was delivered to both the inner and outer surfaces of the ear. The methanol extract (1 mg) was diluted in a 20- μL solution of methanol-chloroform-water at a ratio of 2:1:1. Either the 1-mg sample in vehicles or the vehicle alone (control) was applied topically to the ear approximately 30 min before TPA treatment. Ear thickness was measured using a pocket thickness gauge (Mitsutoyo Co. Ltd., Tokyo, Japan) applied to the tip of the ear. The gauge had a range of 0–9 mm and was graduated at 0.01-mm intervals. It was modified so that the contact surface area was increased, thus decreasing the surface tension.

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

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

Oedema A: oedema was induced by TPA alone (*b*–*a*).

Oedema B: oedema was induced by TPA plus sample (*b'*–*a*).

$$\text{Inhibitory ratio (\%)} = \frac{\text{Oedema A} - \text{Oedema B}}{\text{Oedema A}}$$

Each value was the mean of individual measurements obtained from 4 mice.

6 Two-stage carcinogenesis experiment

The backs of the mice were shaved with electric clippers when they were 7 weeks old. Tumour initiation was accomplished by a single topical application of 50 μg of DMBA. Promotion with 1 μg of TPA, applied twice weekly, was then commenced 1 week after initiation. The experimental group (15 mice) received a methanol extract of *Eucommia* bark (1 mg/mouse) that was diluted in a 100- μL solution of acetone-dimethylsulfoxide-water at a ratio of 8:1:1. The control group (15 mice) received only the 100- μL solution of acetone-dimethylsulfoxide-water at a ratio of 8:1:1. Both the test and control samples were topically applied 30 min before each TPA treatment. DMBA and TPA were dissolved in acetone and a volume of 100 μL was applied to the shaved area using a micropipette. The back of each animal was shaved once a week. The number and diameter of skin tumours were measured on alternate weeks, and the experiment was continued for 20 weeks.

7 Statistical analysis

Statistical analysis was performed using Student's *t*-test and the Mann Whitney *U* exact test.

RESULTS AND DISCUSSION

Various extracts of the dried barks and leaves of *E. ulmoides* were tested for their ability to decrease the intensity of TPA-induced ear oedema (Table 1). It was demonstrated that the dried barks of *E. ulmoides* extract had an inhibitory effect against TPA-induced ear oedema. The methanol extract obtained from the bark of *E. ulmoides* was also more effective than that obtained from the leaves of *E. ulmoides*. The inhibition of TPA-induced inflammation has been demonstrated to be

Table 1 Inhibitory effect of *Eucommia ulmoides* on TPA-induced inflammation in mice

Sample	I.R.
MeOH ext. from leaves of <i>E. ulmoides</i>	29
MeOH ext. from bark of <i>E. ulmoides</i>	80***
Indomethacin	96***

I.R.: Inhibitory ratio at 1.0 mg/ear. *** $p < 0.001$ vs vehicle.

Fig. 1 Inhibitory effect of methanol extract of *Eucommia* bark on the promotion of skin papillomas by TPA in DMBA-initiated mice.

A: Percentage incidence of mice bearing papillomas; B: average numbers of papillomas per mouse, ●, TPA with vehicle; ○, TPA with methanol extract of *Eucommia* bark (1 mg/mouse). $n=15$. * $P<0.01$ vs vehicle.

closely parallel to the inhibition of tumour promotion in two-stage skin carcinogenesis initiated by DMBA and TPA in a mouse model¹⁰. Therefore, we investigated the inhibitory effects of a methanol extract from *Eucommia* bark in a mouse model of two-stage skin carcinogenesis, which was initiated using DMBA and promoted using TPA.

Fig. 1A illustrates the time course of skin tumour formation in the groups treated with DMBA plus TPA with (experimental) or without (control) the methanol extract from *Eucommia* bark. The first tumour appeared during week 4 in the control group and during week 10 in the experimental group. The proportion of tumour-bearing mice at week 20 was 100% in the control group and 33% in the experimental group. Fig. 1B shows the average number of tumours per mouse. The number of tumours per mouse by week 20 was 16.5 in the control group and 1.8 in the experimental group. Overall, treatment with 1.0 mg of methanol extract from *Eucommia* bark was associated with an 89% decrease in the average number of tumours per mouse at week 20. The methanol extract (1 mg/mouse) of the *Eucommia* bark showed the inhibitory effect that was similar in activity to methanol extract (1 mg/mouse) of galangal¹¹, but was more effective than methanol extract (1 mg/mouse) of artichoke flower¹² in two-stage skin carcinogenesis model.

Eucommia bark inhibits lipopolysaccharide (LPS)-induced production of tumour necrosis factor- α (TNF- α) and interleukin-6. Exposure to *Eucommia* bark also decreases inflammation-induced increases in cyclooxygenase-2 (COX-2) levels and the production of prostaglandin-E₂ (PG-E₂) and nitric oxide (NO) in mouse peritoneal macrophages. Furthermore, *Eucommia* bark suppresses the activation of nuclear factor-kappa B (NF- κ B) and caspase-1¹³.

Syringaresinol and its glucosides, namely *O*- β -D-glucoside and di-*O*- β -D-glucoside, as well as lignins were isolated as the major components of *Eucommia* bark^{3,4}. Furthermore, syringaresinol and its glucosides showed strong α,α -diphenyl- β -picrylhydrazyl (DPPH) radical-scavenging activity^{13,14}. Free radicals are directly implicated in the development of cancer by their ability to damage the DNA in a cell's nucleus and weaken the immune system, which normally destroys cancer cells or prevents them from reproducing. In the presence of a strong antioxidant defence system, free radicals are neutralized before they can damage DNA; alternatively, the damage can be detected and repaired before progression. Many antioxidants have been associated with a lower risk of cancer. Therefore, it can be assumed that syringaresinol and its glucosides are potential agents for cancer prevention. Moreover, in a previous study, syringaresinol potently inhibited LPS-induced production of NO, PG-E₂ and TNF- α in macrophages. The expression of inducible NO synthase (iNOS) and COX-2 enzyme was also decreased by syringaresinol in a concentration-dependent manner¹⁵.

This is the first study, as per our knowledge, to report that methanol extracts from *Eucommia* bark inhibited TPA-induced inflammation in mice. Furthermore, these methanol extracts inhibited tumour promotion by TPA following initiation with DMBA. This experiment suggests the potential use of *Eucommia* bark in cancer prevention. The aim of future investigations should be isolation of the active agent and elucidation of the mechanism by which *Eucommia* bark exerts its cancer preventive effects. We will examine the dosage dependence of the *Eucommia* bark.

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要 旨

杜仲樹皮のマウス二段階皮膚発癌実験における腫瘍発現の抑制効果

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癌予防は、公衆衛生分野の重要な問題である。東洋諸国では、杜仲樹皮が強壮や抗高血圧に使用されている。杜仲樹皮は、マウスで TPA によって誘発された炎症を抑制した。我々は、1 mg/mouse の用量で杜仲樹皮メタノールエキ스가、DMBA でイニシエーションをしたマウスの TPA による腫瘍促進抑制を証明した。この結果は、杜仲樹皮の癌予防の可能性を示唆している。

キーワード：杜仲，癌予防，二段階発癌