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Inhibitory Effect of Euphol, a Triterpene Alcohol from the Roots of *Euphorbia kansui*, on Tumour Promotion by 12-*O*-Tetradecanoylphorbol-13-acetate in Two-stage Carcinogenesis in Mouse Skin

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Abstract

The anti-inflammatory activity of euphol, twelve other triterpene alcohols and sitosterol-β-D-glucopyranoside, isolated from the dichloromethane extract of the roots of *Euphorbia kansui*, has been evaluated in mice with inflammation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

TPA (1·7 nmol; 1·0 μg/ear) was dissolved in acetone and 10 μL delivered to the inner and outer surfaces of the right ear of ICR mice. A triterpene alcohol, sterol glucoside or vehicle (20 μL; chloroform–methanol 1 : 1), was applied topically approximately 30 min before each TPA treatment. The ear thickness was measured before treatment and then oedema was measured 6 h after TPA treatment.

For the two-stage carcinogenesis experiment, initiation was accomplished by administration of a single topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA; 195 nmol; 50 μg/mouse) to the shaved backs of mice. Promotion was with 1·7 nmol (1·0 μg) TPA, applied twice weekly to the same shaved area, begun one week after the initiation. Euphol (2·0 μmol; 853 μg), or its vehicle (acetone–dimethylsulphoxide, 9 : 1; 100 μL), was applied topically 30 min before each TPA treatment. The number and diameter of skin tumours were measured every other week for 20 weeks.

All the compounds were found to possess marked inhibitory activity and their 50% inhibitory dose for TPA-induced inflammation was 0·2–1·0 mg/ear. Topical application of euphol (2·0 μmol; 853 μg/mouse) markedly suppressed the tumour-promoting effect of TPA (1·7 nmol; 1·0 μg/mouse) in mouse skin initiated with DMBA.

Naturally occurring triterpenoids often exhibit a variety of biological activities such as anti-inflammatory (Recio et al 1995), anti-HIV (Evers et al 1996; Soler et al 1996), anti-tumour-promoting (Nishino et al 1986; Tokuda et al 1986), ichthyotoxic (Ito et al 1999), and tumour-promoting (Takahashi et al 1999) activities. We have shown that various triterpene alcohols and their derivatives inhibited the tumour-promoting activity of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in two-stage carcinogenesis in mouse skin (Yasukawa & Akihisa 1997).

Although a number of species of the genus *Euphorbia* (Euphorbiaceae) such as *E. aleppica*, *E. characias*, *E. cyparissias*, *E. dendroides*, *E. ebracteolata*, *E. helioscopia*, *E. hirta*, *E. kansui*, *E. lathyris*, *E. myrsinites*, *E. pallasii*, *E. paralias*, *E. pekinensis*, *E. pilosa*, *E. resinifera* and *E. soongarica*, have been used therapeutically around the world, in China *E. kansui* has been characterized best (Stuart 1979; Namba 1994).

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In Chinese traditional medicine the roots of *E. kansui* are used for the treatment of skin oedema, as described in the Dictionary of Chinese Crude Drugs (Chian Su New Medical College 1997). Previous phytochemical investigations on this plant have resulted in the isolation of diterpenes (Uemura et al 1974a, b, 1975a, b; Pan et al 1991; Wu et al 1991; Matsumoto et al 1992), triterpenes (Chen 1982; Ding & Jia 1992), sterols (Ding & Jia 1992) and phenols (Ding & Jia 1992).

The aim of this study was to determine the possible inhibitory effect of triterpene alcohols and a sterol glucoside isolated from *E. kansui* on TPA-induced mouse ear oedema. The 50% inhibitory dose (ID50) of euphol (**1**; Figure 1) was 0·2 mg/ear. To obtain an ID50 value of between 0·2 and 2·0 μmol/ear against TPA-induced inflammatory ear oedema, the dose of test samples used was 2·0 μmol/mouse on two-stage carcinogenesis in mouse skin. Euphol, the main component (approximately 21%) of the extract, markedly inhibited tumour promotion by TPA in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated ICR mice.

Materials and Methods

Instruments

Reverse-phase high-performance liquid chromatography (HPLC) was carried out on octadecyl silica columns (25 cm × 10 mm i.d.), on a Superiorex ODS S-5 μm column (Shiseido Co., Ltd, Tokyo, Japan) (HPLC I) and on a TSK ODS-120A 5 μm column (Toso Co., Tokyo, Japan) (HPLC II), with MeOH (4 mL min^{-1}) as mobile phase. Gas–liquid chromatography (GLC) was performed using a DB-17 fused-silica capillary column (30 m × 0·3 mm i.d., column temp. 275°C). ^{1}H NMR spectra were measured with a JEOL JNM-GSX 400 (400 MHz) spectrometer. Mass spectra were recorded with a Hitachi M-80B double-focusing GC–MS instrument (70 eV) using a direct inlet system.

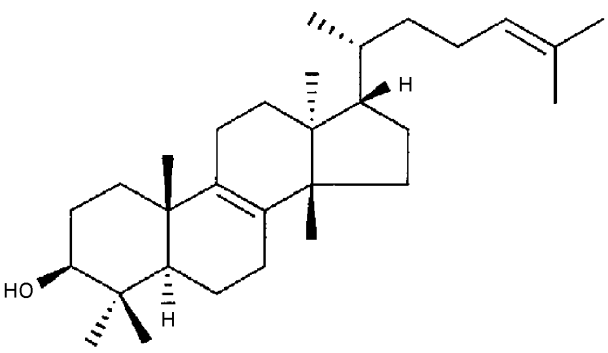

Figure 1. Structure of euphol (**1**).

Chemicals

DMBA and dimethylsulphoxide were purchased from Sigma Chemical (St Louis, MO). TPA was obtained from Chemicals for Cancer Research Inc. (Minneapolis, MN).

Material

Dried roots of *Euphorbia kansui* Liou. were purchased from Kinokuniya Kan-Yaku Kyoku Co. (Tokyo, Japan).

Isolation procedures

The dried roots of *E. kansui* (1 kg) were extracted three times for three days with CH_2Cl_2 at room temperature to give 22·5-g extract. The extract was subjected to column chromatography on silica gel (500 g) using an *n*-hexane–ethyl acetate gradient of 1 : 0–0 : 1 and methanol, which yielded six fractions A–F. Further column chromatography of the second least-polar fraction B on silica gel yielded purified fraction B (5·4 g) which was acetylated in Ac_2O–pyridine at room temperature over night to give the acetate fraction B (4·7 g). Recrystallization of the fraction B acetate from methanol gave 3·3 g crystals, constituted with euphol (**1**; see Figure 1 for the structure) acetate, and filtrate portions (1·2 g). Preparative HPLC (I and II) of the filtrate portion eventually yielded twelve known triterpene alcohols (**2**–**13**) as the acetyl derivatives (Table 1). Identification of these compounds was performed by direct comparison of the chromatographic (HPLC, GLC) and spectroscopic (EI–MS, ^{1}H NMR) data with respective authentic compounds (Akihisa et al 1997). Alkaline hydrolysis (5% KOH in methanol, room temperature overnight) of the isolated triterpene acetates yielded free alcohols which were used for bioassay. The most-polar fraction F (0·3 g) was subjected to preparative HPLC I which yielded sitosterol-β-D-glucopyranoside (**14**; 23 mg) (CI–MS: m/z 594·4788; required for $C_{35}H_{64}O_6N$ $[M+NH_4]^+$: 594·4730). Identification of compound **14** was performed by ^{1}H NMR spectral comparison with the literature data for the corresponding compound (Kojima et al 1990).

Animals

Female ICR mice (7-weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan). The ani-

Table 1. Composition (%) of the triterpene alcohol fraction, and inhibitory effect of triterpene alcohols and a sterol glucoside isolated from *Euphorbia kansui* roots, and reference compounds.

Code	Compound (systematic name)	Composition (%)[a]	ID50	
			mg/ear	μmol/ear
Triterpene alcohol				
1	Euphol (eupha-8,24-dien-3β-ol)	87·0	0·2	0·5
2	Tirucallol (tirucalla-8,24-dien-3β-ol)	9·6	0·4	0·9
3	24-Methylenecycloartanol (24-methylenecycloartan-3β-ol)	0·9	0·2	0·5
4	Cycloartenol (cycloart-24-en-3β-ol)	0·7	0·3	0·8
5	24-Methylene-24-dihydroparkeol [24-methylenelanost-9(11)-en-3β-ol]	0·7	0·4	0·9
6	Dammaradienol (dammara-20,24-dien-3β-ol)	0·3	0·8	1·9
7	Isotirucallol [(20*S*)-dammara-13(17),24-dien-3β-ol]	0·3	0·3	0·7
8	Isoeuphol [(20*R*)-dammara-13(17),24-dien-3β-ol]	0·2	0·3	0·7
9	Butyrospermol (eupha-7,24-dien-3β-ol)	0·1	0·6	1·4
10	Lupeol [lup-20(29)-en-3β-ol]	0·1	0·6	1·4
11	β-Amyrin (olean-12-en-3β-ol)	< 0·1	0·4	0·9
12	24-Methylene-24-dihydrolanosterol (24-methylenelanost-8-en-3β-ol)	< 0·1	nd	nd
13	Δ^7-Tirucallol (tirucalla-7,24-dien-3β-ol)	< 0·1	0·8	1·9
Sterol glucoside				
14	Sitosterol-β-D-glucopyranoside [(24*R*)-stigmast-5-en-3β-ol-β-D-glucopyranoside]		1·0	1·7
Reference compound				
	Quercetin		1·6	5·3
	Indomethacin		0·3	0·8

[a]Percentage composition of the triterpene alcohol fraction determined based on HPLC and GLC.

mals were housed in an air-conditioned specific pathogen free room (22–23 °C), lit from 0800 to 2000 h. Food and water were freely available.

Assay of TPA-induced inflammatory ear oedema

TPA (1·7 nmol; 1·0 μg/ear) dissolved in acetone (20 μL) was applied to the right ear only 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, or its vehicle (20 μL; chloroform–methanol 1 : 1) as a control, was applied topically approximately 30 min before each TPA treatment. For thickness determinations, 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 the tensions, was applied to the tip of the ear.

The ear thickness was measured before treatment (a), and then oedema was measured 6 h after TPA treatment (b: TPA alone; b′: TPA plus sample). The following values were then calculated:

Oedema A :

$$\text{oedema induced by TPA alone } (b - a) \quad (1)$$

Oedema B :

$$\text{oedema induced by TPA plus sample } (b' - a) \quad (2)$$

$$\text{Inhibitory ratio } (\%) = ((\text{Oedema A} - \text{Oedema B})/\text{Oedema A}) \times 100 \quad (3)$$

Each value used was the mean of individual determinations from five mice. The 50% inhibitory dose (ID50) values were determined by probit-graphic interpolation for four dose levels.

Two-stage carcinogenesis experiment
The backs of mice (7-weeks old) were shaved with electric clippers. Initiation was accomplished by a single topical application of 195 nmol (50 μg) DMBA. Promotion with 1·7 nmol (1·0 μg) TPA, applied twice weekly, was begun one week after the initiation. Euphol (2·0 μmol; 853 μg), or its vehicle (acetone–dimethylsulphoxide 9 : 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 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 tumours were measured every other week, and the experiment was continued for 20 weeks. The experimental and appropriate control groups each consisted of 15 mice.

Statistical analysis
Statistical analysis was by Student's *t*-test.

Results

Effects of triterpene alcohols and a sterol glycoside from E. kansui *on TPA-induced ear oedema*
The 13 triterpene alcohols (**1**–**13**) and a sterol glucoside (**14**) isolated from the dichloromethane extract of *E. kansui* roots were examined for their inhibitory effects on TPA-induced inflammation in mice. All of the compounds markedly inhibited the TPA-induced inflammation with 0·2–1·0 mg/ear, being the 50% inhibitory dose. Euphol (**1**), the most predominant triterpene alcohol constituent, and 24-methylenecycloartanol (**3**) exhibited the strongest inhibitory effect (0·2 mg/ear) (Table 1).

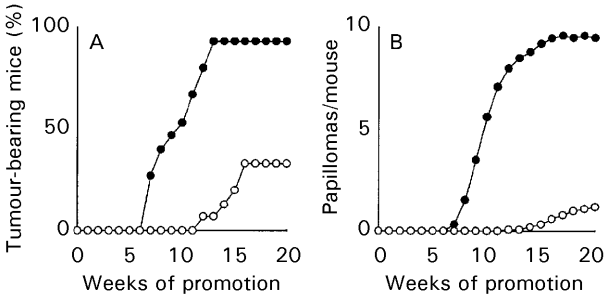

Figure 2. Inhibitory effect of euphol on the promotion of skin papillomas by TPA in DMBA-initiated mice. From one week after initiation by a single topical application of 195 nmol (50 μg) DMBA, 1·7 nmol (1·0 μg) TPA was applied twice weekly. Topical application of euphol (2·0 μmol; 853 μg/mouse) and vehicle was performed 30 min before TPA treatment. Data are expressed as percentage of mice bearing papillomas (A) and as an average number of papillomas per mouse (B). ● TPA with vehicle alone, ○ TPA with euphol.

Inhibitory effect of euphol on the tumour-promoting activity of TPA
Figure 2A shows the time courses of skin tumour formation in the group treated with DMBA plus TPA, with or without euphol. The first tumour appeared at week 7 in the group treated with DMBA plus TPA. In the group treated with DMBA plus TPA and euphol, the first tumour appeared at week 12. The proportion of tumour-bearing mice treated with DMBA plus TPA was 93% at week 20, whereas the proportion in the groups treated with DMBA plus TPA and euphol was 20%. Figure 2B shows the average number of tumours per mouse. The group treated with DMBA plus TPA produced 9·7 tumours per mouse at week 20, whereas the group treated with DMBA plus TPA and euphol had 1·0 tumours per mouse. Euphol treatment resulted in a 90% reduction in the average number of tumours per mouse at week 20.

Discussion

The dichloromethane extract of the roots of *E. kansui* inhibited the inflammatory ear oedema induced by TPA. Triterpene alcohols and a sterol glucoside isolated from the extract markedly inhibited the inflammation. Euphol was more effective than the other components. Ingenane-type diterpenes isolated from *E. peplus* have been reported to possess skin irritant and tumour-promoting effects in mouse skin (Krauter et al 1996; Zayed et al 1998). The biochemical and biological properties of these compounds have been shown to be similar with those of TPA-type tumour promoter, phorbol esters, teleocidins and aplysiatoxins (Fujiki et al 1989; Krauter et al 1996). Although these diterpene derivatives were found in *E. kansui*, the roots of *E. kansui* have been used as an anti-oedema remedy for skin. This might explain why euphol and other triterpene alcohols contained in the *E. kansui* roots inhibited irritancy and tumour-promoting effects of ingenane-type diterpenes.

In this study, we have shown that euphol, an euphane-type triterpene alcohol, possesses anti-tumour-promoting activity. Various triterpene derivatives of the other skeletal-types, e.g. tarax-

astane-, oleanae-, ursane-, lupane-, lanostane- and multiflorane-types, have been demonstrated to inhibit the tumour-promoting activity of TPA (Yasukawa et al 1991, 1994a, b, 1995, 1996a, b, 1998a, b; Kasahara et al 1994; Kaminaga et al 1996). Comparing the inhibitory activity of euphol with other triterpenes, euphol was less effective than di- and trihydroxy triterpenes, faradiol and heliantriol C (Yasukawa et al 1998a), and triterpene acids, pachymic acid, 3-*O*-acetyl-16α-hydroxy-trametenolic acid and poricoic acid B (Kaminaga et al 1996). However, the inhibitory activity of euphol was similar to lupeol (Yasukawa et al 1995), karounidiol (Yasukawa et al 1994a), and taraxasterol (Yasukawa et al 1996b). In comparison with the other naturally occurring compounds, euphol was more inhibitory than flavonoids (Yasukawa et al 1990) and sterols (Yasukawa et al 1994b, 1996a). Contrary to the above, an iridal-type triterpene has been found to promote tumour in DMBA-initiated mice (Takahashi et al 1999). It is, therefore, conceivable that there are two types of triterpenes, ones with tumour-promoting activity and others with anti-tumour-promoting activity.

We have demonstrated previously that extracts from edible plants and fungi inhibited TPA-induced inflammation in mice. Sterols and triterpenes separated from an edible mushroom *Hypsizigus marmoreus* (Yasukawa et al 1994a), safflower (Kasahara et al 1994), an edible alga *Chlorella vulgaris* (Yasukawa et al 1996a) and stevia (Yasukawa et al 1993) have been identified as the active compounds. Furthermore, these sterols and triterpenes have been shown to inhibit tumour promotion during two-stage carcinogenesis in mouse skin. Other researchers have reported the inhibitory activity of triterpenes on tumour promotion (Nishino et al 1986; Tokuda et al 1986, 1991; Konoshima et al 1992, 1994, 1995; Yu et al 1992, 1995; Diallo et al 1995). Many sterols and triterpenes, widely distributed in edible plants and fungi, inhibit the tumour-promoting activity of TPA in mouse skin, and this suggests that they may be important for the chemoprevention of cancer.

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