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Preparative Isolation and Gas Chromatography-Mass Spectrometry Analysis of Triterpenoids in Kansui Radix

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ABSTRACT

Two triterpenoids, euphol and tirucallol were isolated from Kansui Radix (*Euphorbia kansui* Liou, Euphorbiaceae) by a modified isolation method. These constituents were used as marker substances for quality control of Kansui Radix using a home-developed gas chromatography-mass spectrometry (GC-MS) method.

The linear calibration ranges were 50.0-400.0 μg/mL (r=0.9988) for euphol and 20.0-200.0 μg/mL (r=0.9976) for tirucallol. The relative standard deviations of two marker substances for intraday and interday analyses were 0.90-3.07% and 3.93-8.17%, respectively. Recoveries were euphol 98.3±1.2%, and tirucallol 84.8±.0%. The contents of two marker substances in seven crude drugs of Kansui Radix were euphol 0.10-0.19%, and tirucallol 0.05-0.07%.

Key words: pharmaceutical analysis, Kansui Radix, tirucallol, euphol, GC-MS

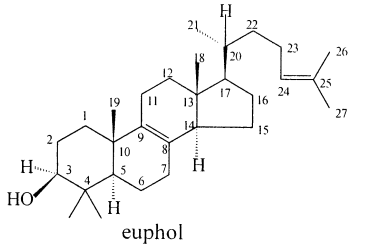
INTRODUCTION

Traditional Chinese herbal medicines, which have been used for centuries, require effective chromatographic methods to ensure quality control. In our laboratory, we have developed several high-performance liquid chromatographic (HPLC)[1-4] and capillary electrophoresis (CE)[5-8] methods for the determination of marker constituents in traditional Chinese medicines.

Kansui Radix is the dried root of *Euphorbia kansui* Liou (Euphorbiaceae) and a toxic herbal medicine. It is administered for the treatments of anasarca, ascites, tympanitis, hernia hydrocele and dysuria[9].

Several diterpenoids[10-13] and triterpenoids[14-16] have been isolated from this plant. Many triterpenoids are important because of their biological activities. The troublesome process of separating a pure compound from crude extracts results in difficulty for determining the quantities and characterizing the active ingredients. Because of the low sensitivity at the detection wavelength and high detection limit due to the background interference of triterpenoid, the popular method for quantitative analysis, HPLC, is not suitable.

Euphol is the major triterpenoid of *Euphorbia kansui*, and its diastereomer: tirucallol also contained in this plant[14-16]. The chemical structures of two constituents are shown in Figure 1. Euphol is also found in other plants of the same genus, such as *E. antiquorum* and *E. broteri*. Tirucallol, but not euphol, was found to have strong antiviral activity. The difference of cytotoxicities between the two compounds was correlated with the stereochemistry of the proton on C-20[16]. In this study, the separation of these two constituents in



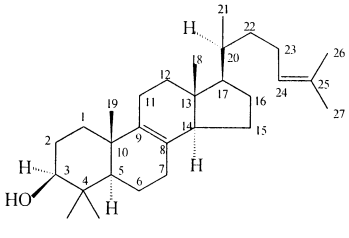


Figure 1. The chemical structures of euphol and tirucallol.

Euphorbia kansui was carried out by using a column packed with silver nitrate coated silica gel. A rapid and simple gas chromatography-mass spectrometry (GC-MS) method for routine quantitative analysis of Kansui Radix was developed.

MATERIALS AND METHODS

I. *Materials*

The roots of *Euphorbia kansui* 2.0 kg and seven crude drug samples of Kansui Radix (ca. 40 g) were obtained from local markets in Taipei. The authenticity of these samples was verified using the voucher specimen deposited in the National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, Republic of China.

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II. *Apparatus*

Melting points measured with a Fisher-Johns melting-point apparatus were used directly. Optical rotation was measured with a Jasco DIP-1000 polarimeter. NMR spectra were recorded on a Bruker AM-300 WB/DMX-500 SB FT-NMR spectrometer. A Hewlett-Packard 5890 gas chromatograph with an HP 5973 mass selective detector and an HP 6890 series injector was employed.

III. *Working Standards and Reagents*

Euphol and tirucallol were isolated from the root of *Euphorbia kansui*. Cholesterol was purchased from Merck (Darmstadt, Germany). Chloroform, methanol, n-hexane, acetone, toluene, ethylacetate and dichlormethane were analytical grade. Column chromatography was carried out with Silica gel 60 (70-230 mesh, Merck).

IV. *Extraction, Separation and Characterization*

The root of *Euphorbia kansui* (2.0 kg) was cut into small pieces and extracted five times with chloroform (15 l) by refluxing at 60˚C, one hour each time. The chloroform was removed by evaporation under reduced pressure, and the resulting precipitate removed by filtration. The filtrate, after concentration, was subjected to a silica gel medium pressure column chromatography using n-hexane containing increasing amounts of toluene (0-100 %). The solvent polarity was further increased using dichloromethane, ethyl acetate and methanol, sequentially. The combined triterpenoid fractions were chromatographed again over silica gel with toluene. The residue of the eluent was re-crystallized using n-hexane yielded euphol. The mother liquid was chromatographed over a 10 % silver nitrate-silica gel column (n-hexane) and eluted with n-hexane-dichloromethane (1:2) to give tirucallol and euphol.

V. *GC-MS System*

The column was a 30 m×0.25 mm I.D. fused silica capillary cross-linked column with 5 % phenylmethylsilicone phase, 0.25 μm film thickness (HP-5MS; HP). Helium was used as the carrier gas with a constant flow of 1.0 mL/min. The column head pressure was set at 10.5 p.s.i. Temperature conditions were as follows: the initial temperature was 200˚C for 2 min, then increased at 10˚C /min by linear to 300˚C, and held for 4 min. A 1-μL volume sample was injected using the splitless mode. Full-scan mass spectra were collected between 40 and 550 amu at 2.89 scan/s. The MS was operated in the electron impact (EI) ionization mode with an electron energy of 70 eV. The ion source and quadrupole temperatures were maintained at 230 and 150˚C, respectively. For euphol and tirucallol, ions with *m/z* 393 and 411 were monitored, and for cholesterol, ions with *m/z* 275 and 386 were monitored.

VI. *Preparation of Standard Solutions*

To prepare standard solutions (containing euphol and tirucallol), an appropriate amount of internal standard solution was added to an accurately weighed amount of euphol and tirucallol standard which was dissolved in methanol for GC-MS. The various concentrations were within the range 50.0-400.0 and 20.0-200.0 μg/mL, respectively. Calibration graphs were plotted subsequently for linear regression analysis of the peak area ratios with concentrations.

VII. *Preparation of Sample Solution*

(I) *Solvent Extraction*

Twenty grams of Kansui Radix was cut into pieces and mixed well. Two grams of the sample was extracted three times with 10 mL of methanol, acetone, dichlormethane, n-hexane and ethyl acetate separately by reflux, for 30 min each. The extracts were filtered into a volumetric flask, methanol was added to 25 mL, respectively. This solution was filtered through a 0.45 μm syringe filter (Whatman) before use.

(II) *Sample Determination*

Four gram of each Kansui Radix sample was extracted two times with 20 mL of acetone in an ultrasonic bath, filtered, and then made to 50 mL with acetone. An aliquot of 4.5 mL of the above solution and 0.5 mL of cholesterol solution (0.5 mg/mL) was placed into a 5 mL volumetric flask. This solution was filtered through a 0.45 μm syringe filter (Whatman) before use.

VIII. *Recovery Studies*

Three different concentrations of markers: 100.0, 150.0 and 200.0 μg/mL for euphol and 40.0, 60.0 and 80.0 μg/mL for tirucallol were added to sample solution, respectively. To each solution, a suitable amount of internal standard was added to yield a final concentration of 50.0 μg/mL of cholesterol. All samples were filtered through a 0.45 μm syringe filter (Whatman) and injected for GC-MS analysis to calculate the concentration of euphol and tirucallol from their calibration graphs.

Recovery (%) = (Amount measured / Amount added)×100 %

RESULTS AND DISCUSSION

I. *Isolation of Working Standard*

The chloroform extract of Kansui Radix was repeatedly chromatographed on a silica gel column to give euphol. References 14-16 show the isolation of euphol and tirucallol using repeat column chromatography and recrystallization. In this study, a 10% silver nitrate-silica gel column was used for the separation of these two optical isomers. This modified

method was easier and more time saving than silica gel without silver nitrate coating.

Euphol

White needles (n-hexane), mp 105˚C, $[\alpha]_D^{25}$ + 25.1˚ (c=0.12, n-hexane). EIMS (70 eV) *m/z*: 393, 411, 426 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 5.07 (1H, *t*, J=7.1Hz, H-24), 3.22 (1H, *dd*, *J*=4.5, 11.7 Hz, H-3),1.66, 1.58, 0.98, 0.93, 0.86, 0.78, 0.73 (each 3H, *s*, CH_3×7), 0.84 (3H, *d*, *J*=6.6 Hz, CH_3). The ^{13}C-NMR spectrum data are given in Table 1.

Tirucallol

White needles (n-hexane), mp 130˚C, $[\alpha]_D^{25}$ – (18.4˚ (c= 0.09, n-hexane). EIMS (70 eV) *m/z*: 393, 411, 426(M^+). ^{1}H-NMR ($CDCl_3$) δ: 5.08 (1H, *t*, *J*=7.1 Hz , H-24), 3.22 (1H, *dd*, *J*=4.5, 11.7 Hz, H-3), 1.66, 1.58, 0.98, 0.93, 0.84, 0.78, 0.73 (each 3H, *s*, CH_3×7), 0.89 (3H, *d*, *J*=6.4 Hz, CH_3). The ^{13}C-NMR spectrum data are given in Table 1.

II. *Solvent Extraction*

The extraction yields of two constituents using different solvents are shown in Table 2. With acetone the two constituents yielded the best extraction rates. Euphol and tirucallol yielded over 90.0 % in the first extraction by acetone. Therefore, two extractions with acetone used throughout this study.

III. *Calibration Graphs for Euphol and Tirucallol*

Calibration graphs were constructed in the range 50.0-400.0 μg/mL for euphol and 20.0-200.0 μg/mL for tirucallol. The regression equations of these curves and their correlation coefficients were calculated as follows: euphol, y = 17.12x + 24.71 (r=0.9988) and tirucallol, y = 15.44x + 11.35 (r=0.9976). The results showed good linear relationships between the peak area ratio and the concentration.

The full scan spectrum (from m/z 40 to 550) of euphol is shown in Figure 2. Euphol yielded a molecular ion at m/z 426 and major fragment ions at 411 and 393. The spectrum of tirucallol was similar to that of euphol. The present study used the selected ion monitoring (SIM) mode for quantitative analysis. The monitored ions of euphol and tirucallol were the same, and were m/z 393 and 411.

IV. *Suitability Tests*

To assess the precision of this method, we injected standard solutions of euphol and tirucallol, respectively, five times on the same day and over a 5-day period. The coefficient variations of intraday and interday studies were 0.90-3.07 % and 3.93-8.17 %, respectively (Table 3). The recoveries of euphol and tirucallol ranged from 83.4 to 99.3 %. The R.S.D.s of recoveries of two constituents ranged between 1.1-1.2 % (Table 4).

Table 1. The ^{13}C-NMR spectra data (ppm) of euphol and tirucallol (in $CDCl_3$)

Euphol				Tirucallol			
C	ppm	C	ppm	C	ppm	C	ppm
1	35.23	16	29.74	1	35.23	16	29.81
2	27.65	17	49.61	2	27.65	17	49.09
3	79.00	18	15.52	3	78.99	18	15.42
4	38.92	19	20.13	4	38.93	19	20.13
5	50.94	20	35.86	5	50.93	20	35.91
6	18.93	21	18.91	6	18.93	21	18.67
7	27.92	22	35.40	7	27.92	22	37.25
8	133.53	23	24.74	8	133.52	23	24.92
9	134.00	24	125.20	9	134.04	24	125.24
10	37.25	25	130.87	10	37.25	25	130.91
11	21.51	26	17.68	11	21.45	26	17.62
12	28.13	27	25.74	12	28.04	27	25.71
13	44.09	28	24.46	13	44.08	28	24.36
14	50.01	29	28.04	14	49.93	29	27.92
15	30.88	30	15.60	15	30.76	30	15.51

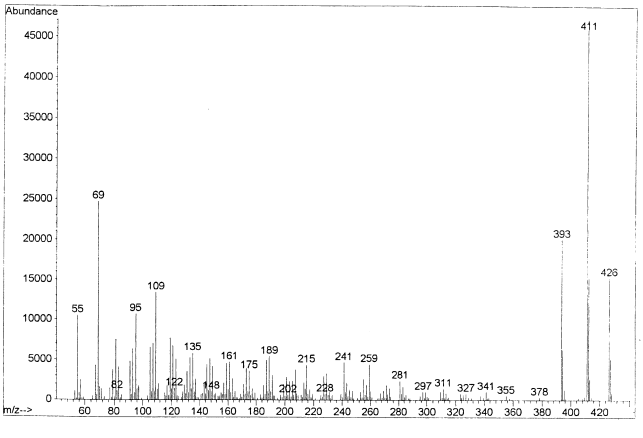

Figure 2. Mass spectrum from m/z 40 to 440 of euphol.

Table 2. The relative extraction rate of euphol and tirucallol in Kansui Radix

	Euphol (%)				Tirucallol (%)			
	Times			Total	Times			Total
Solvent	1	2	3		1	2	3	
n-Hexane	82.9	3.4	0	86.3	75.3	2.1	0	77.4
Dichlormethane	55.8	8.6	0.5	65.0	44.9	5.3	0	50.2
Ethyl acetate	77.6	2.0	0	79.6	69.1	1.2	0	70.4
Acetone	99.0	1.0	0	100.0	99.6	0.4	0	100.0
Methanol	69.8	6.6	0.2	76.7	64.6	4.1	0	68.7

Table 3. Intraday and interday analytical precisions of euphol and tirucallol ([a] n=5)

Constituents	Concentration (μg/mL)	Intraday (R.S.D., %)[a]	Interday (R.S.D., %)[a]
Euphol	50.0	1.45	3.96
	200.0	2.13	7.46
	400.0	3.07	8.17
Tirucallol	20.0	1.09	3.93
	80.0	0.90	6.27
	200.0	1.69	5.98

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Table 4. Recoveries of euphol and tirucallol from Kansui Radix ([a] n=3)

Constituents	Added (μg/mL)	Measured (mean[a], μg/mL)	Recovery (mean[a], %)	Mean±S.D. (%)	R.S.D. (%)
Euphol	100.0	96.6	96.6	98.3±1.2	1.2
	150.0	148.5	99.0		
	200.0	198.6	99.3		
Tirucallol	40.0	34.1	85.3	84.8±1.0	1.1
	60.0	50.0	83.4		
	80.0	68.5	85.6		

V. *Determination of Euphol and Tirucallol in Kansui Radix*

The retention time of two constituents, euphol and tirucallol, and internal standard (cholesterol) were 14.3, 14.8 and 13.0 min, respectively (Figure 3). As the sample solutions were injected directly and analyzed, the results were as good as those obtained and the analysis could be completed within 15 min. The comparison graph of contents of euphol and tirucallol in 7 crude drugs of Kansui Radix is shown in Figure 4. The contents of euphol and tirucallol in crude drugs ranged between 0.10-0.19 % and 0.05-0.07 %. The contents of euphol were much greater than tirucallol. Although euphol did not exhibit antiviral activity, euphol was the major constituent in Kansui Radix and could be used as a marker for quality control.

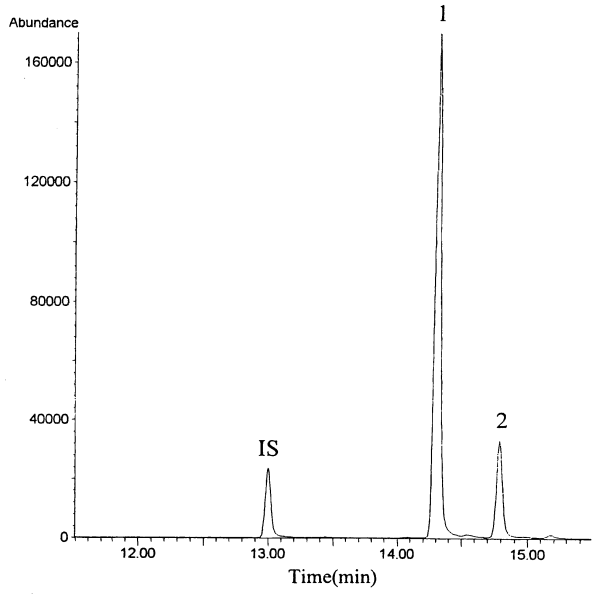

Figure 3. Total ion chromatogram of Kansui Radix. 1: euphol, 2: tirucallol. IS=cholesterol.

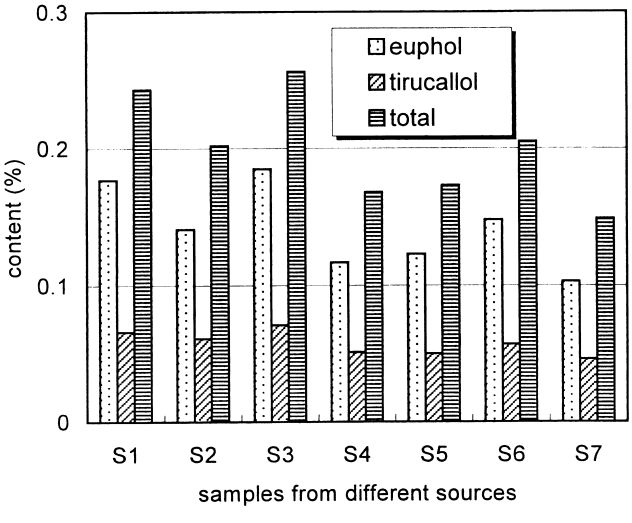

Figure 4. The contents of euphol and tirucallol in seven commercial samples of Kansui Radix.

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甘遂藥材中Triterpenoid成分之分離及氣相層析質譜儀定量研究

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摘　要

本研究針對甘遂藥材中triterpenoid成分，分離純化而得euphol及tirucallol二成分作為指標成分，利用GC-MS探討其定量方法，並調查市售甘遂藥材之指標成分含量。

GC-MS使用毛細管柱（HP-5MS），以euphol（50.0~400.0 μg/mL）及tirucallol（20.0~200.0 μg/mL）做為對照用標準品。其線性迴歸方程式及相關係數（r）分別為euphol：y = 17.12x + 24.71（r = 0.9988）；tirucallol：y = 15.44x + 11.35 (r = 0.9976)，均呈良好線性關係。二種指標成分之回收率，分別為euphol 98.3 ± 1.2%，tirucallol 84.8 ± 1.2%。同日內及異日間相對標準偏差試驗，其同日內為0.90~3.07 %，異日間為3.93~8.17%。

測定七件市售甘遂藥材中二種指標成分之含量。結果euphol之含量平均為0.14%（0.10~0.19%）；tirucallol之含量平均為0.06%（0.05~0.07%）。

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