# Metabonomic approach to Molecular Mechanism of Detoxic Effect of Compatibility of Traditional Chinese Medicine Part I : Toxic Components of *Euphorbia kansui*


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**Abstract:**

The roots of *Euphorbia kansui* (EK) and the fruits of *Zizyphus jujuba* (ZJ) have been clinically used in traditional Chinese medicine (TCM). According to the TCM theory, EK has been frequently used together with ZJ as a drug-pair (named as compatibility), or processed with vinegar (VEK) in many TCM prescriptions to lower its toxicity. Our previous work confirmed the toxicity of water extract of EK, and explored the detoxic effect of VEK and a combination use of EK and ZJ using a NMR-based metabonomic approach. In the present study, to further clarify the toxic and components in water extract of EK, isolation and structure elucidation were conducted and 8 compounds were obtained. Their structures were identified as triterpenes Euphol (**1**), (3β,11β)-3,11-dihydroxylanosta-8,24-dien-7-one (**8**) and ingenol-typed diterpene esters 3-O-benzoyl-20-deoxyingenol (**2**), 5-O-benzoyl-20-deoxyingenol (**3**), 3-*O*-(2′-*E*,4′-*Z*-decadienoyl)-20-*O*-acetylingenol (**4**), 3-*O*-(2′-*E*,4′-*E*-decadienoyl)-20-*O*- acetylingenol (**5**), 20-*O*-(2′-*E*,4′-*Z*-decadienoyl)ingenol (**6**), 20-*O*-(2′-*E*,4′-*E*- decadienoyl)ingenol (**7**) by physiochemical means.



**Keywords:** Euphorbia kansui, traditional Chinese medicine, diterpenes, isolation, metabonomics


## 1. Introduction

The roots of *Euphorbia kansui* T. N. Liou ex T. P. Wang (Euphorbiae kansui radix, Euphorbiaceae), Chinese name ‘Kansui’, is one of the very important herbal drugs listed in Chinese Pharmacopoeia [1a]. As a drastic hydragogue, *Euphorbia kansui* (EK) possesses medical functions of removing water retention, subduing swelling and dissolving lumps and so on. It is reported that the ether, ethyl acetate and ethanol extracts of EK possessed toxicity, and the main toxic components of these extracts are diterpenes [2]. According to the traditional Chinese medicine (TCM) theory, to reduce the toxicity of EK, vinegar processed EK (VEK) has been commonly used and the vinegar processing did reduce the toxicity of EK [3].

In the TCM theory, a combination of herbs and constituents usually attribute to synergism

therapeutic and pharmacological effects, reduction side effects or toxicities, which termed "TCM compatibility". The compatibility is being received increasing attention at the present time. The fruits of *Zizyphus jujuba* (ZJ, Ziziphi fructus), also listed in Chinese Pharmacopoeia [1b] is often prescribed together with EK as a drug pair to lower the toxicity of EK in a number of prescriptions. It is reported that the triterpenoids of ZJ reduced the toxicity of ethanol extract of EK [4]. The important thing is, the water extracts of EK or VEK showed no toxicity [3], however, water decoctions of EK or VEK are commonly used in TCM clinic and considered to have toxicity in TCM. Our previous study shown that when rats were co-administered with water extract of EK and water extract of ZJ exhibited a quite unique de-toxic effect, toxic symptom of EK was lower and prior to that administered with water extract of EK vinegar-treated only (Fig. 1).

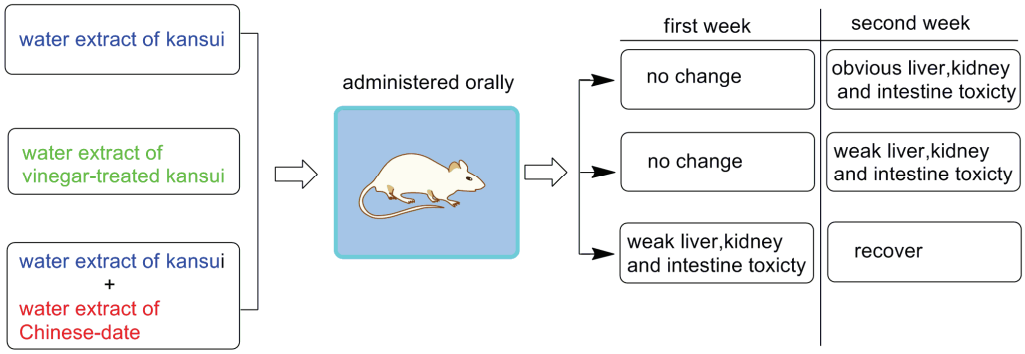
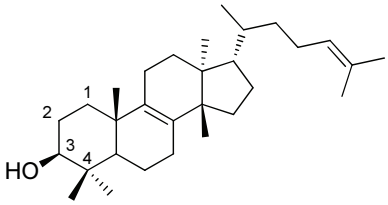



Fig. 1

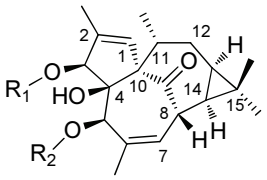
In order to understand the Traditional Chinese Medicine Formulations scientifically and clarify how the detoxicant of ZJ interacts with toxicant of EK to reduce side effects, especially the interactions of components contained in EK and ZJ, we first to isolate the toxicants in the water extract of kansui.

## 2. Results and discussion

The water extract of roots of EK was subjected to a series of chromatographic separations and resulted in the isolation of eight reported compounds: triterpene-type Euphol (**1**) [5] and ingenol-typed diterpene esters 3-O-benzoyl-20-deoxyingenol (**2**) [6], 5-O-benzoyl-20-deoxyingenol (**3**) [6], 3-*O*-(2′-*E*,4′-*Z*-decadienoyl)-20-*O*-acetylingenol (**4**) [7], 3-*O*-(2′-*E*,4′-*E*-decadienoyl)-20-*O*-acetylingenol (**5**) [8], 20-*O*-(2′-*E*,4′-*Z*-decadienoyl)ingenol (**6**) [8], 20-*O*-(2′-*E*,4′-*E*-decadienoyl) ingenol (**7**) [8], (3β,11β)-3,11-dihydroxylanosta-8,24-dien-7-one (**8**) [9]. The structures of these known compounds were determined by analyzing their spectroscopic data and comparison with reported values in the literature.

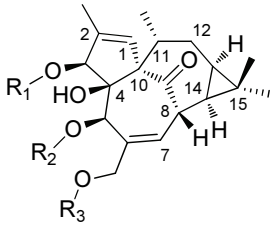


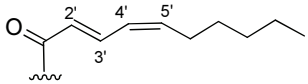
**1** Euphol

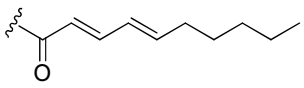


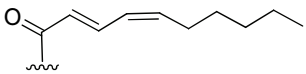
**2** $R_1$ = benzoyl, $R_2$ = H

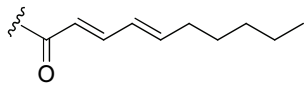
**3** $R_1$ = H, $R_2$ = benzoyl

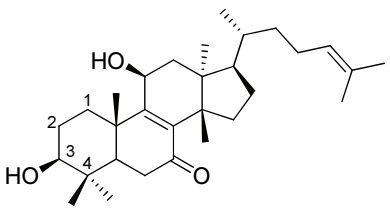


**4** $R_1$ =  $R_2$ = H, $R_3$ = Ac

**5** $R_1$ =  $R_2$ = H, $R_3$ = Ac

**6** $R_1$ = H, $R_2$ = Ac, $R_3$ = 

**7** $R_1$ = H, $R_2$ = Ac, $R_3$ = 



**8**

Here, take compounds **6** and **7** as examples for the discussion of the structure identification. Ingenol-type diterpenes are very difficult to isolate due to their structural similarity especial the long carbon-chain substitutes which just shown *E* and *Z* configurational difference. We got compounds **6** and **7** from subfraction **Fr 10-4** by semipreparitive HPLC with a reverse phase column and normal phase preparative TLC (HPLC spectrum shown as Fig. 2).

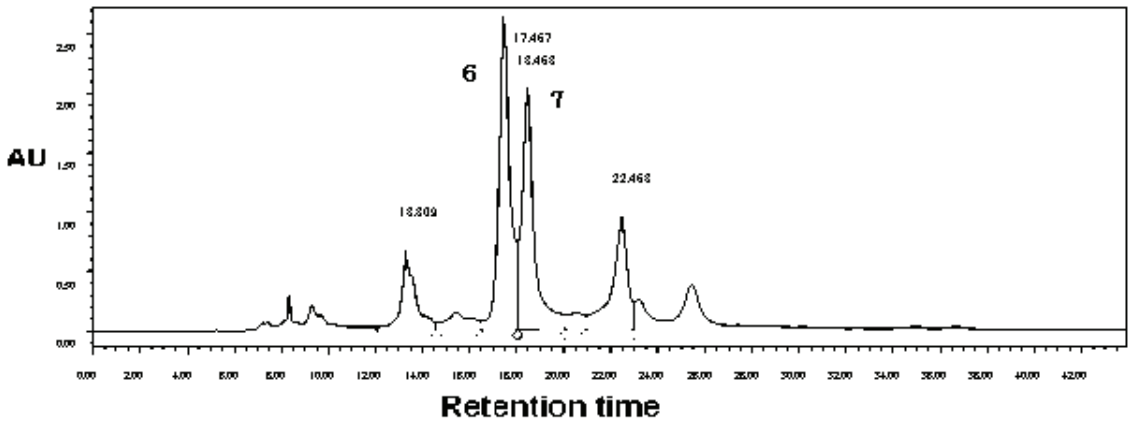



Fig. 2

$^{13}$C-NMR spectrum of **6** showed 30 carbon atoms, and chemical shifts suggested the presence of a carbonyl, an ester carbonyl, and four oxygen-bearing carbon atoms that corresponding to Ingenol-type diterpenes esters. Meanwhile, most of the diterpenes that have a 2,4-decadienoyloxy

group isolated from *Euphorbia* species possess an *E,Z*- or *Z,E*-configuration in the double bonds of the ester residue, In compounds with the *E,Z*-configuration [7,10], the coupling constants are normally $J_{2',3'}$ = 15 Hz, $J_{4',5'}$ = 11 Hz, and H-3′ appears at about δ = 7.68 as a double doublet ($J_{2',3'}$ = 15 Hz, $J_{3',4'}$ = 11 Hz). In compounds with the *Z,E*-configuration [11], the coupling constants are normally $J_{2',3'}$ = 11 Hz, $J_{4',5'}$ = 15 Hz, and the H-3′ signal resonated at about δ = 7.40, moving upfield by about 0.3 ppm compared with those with the *E,Z*-configuration, and appearing as a double doublet ($J_{2',3'}$ = 15 Hz, $J_{3',4'}$ = 11 Hz). In the $^1$H-NMR spectrum of **6**, the H-2′ signal appeared at δ 5.85 as a doublet ($J_{2',3'}$ = 15.2 Hz), and the H-3′ signal was found at δ 7.60 as a double doublet ($J_{2',3'}$ = 15.2 Hz, $J_{3',4'}$ = 11.0 Hz) corresponding to a 2′,3′-trans double bond. Thus, compound **6** was concluded to be 20-*O*-(2′-*E*,4′-*Z*-decadienoyl)ingenol.

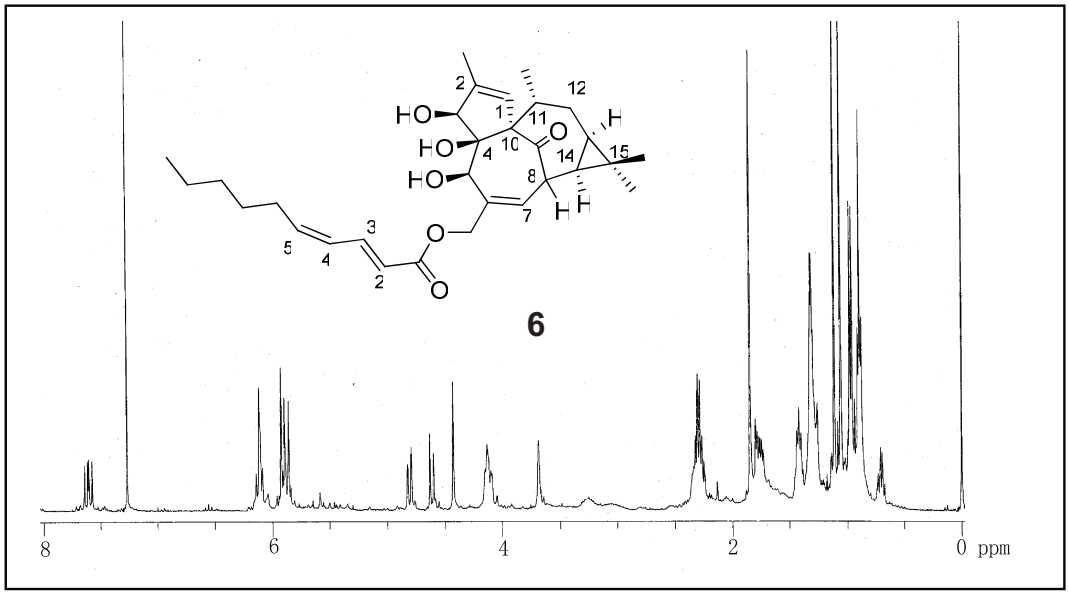



Fig. 3 $^1$H-NMR($CDCl_3$) of compound **6**

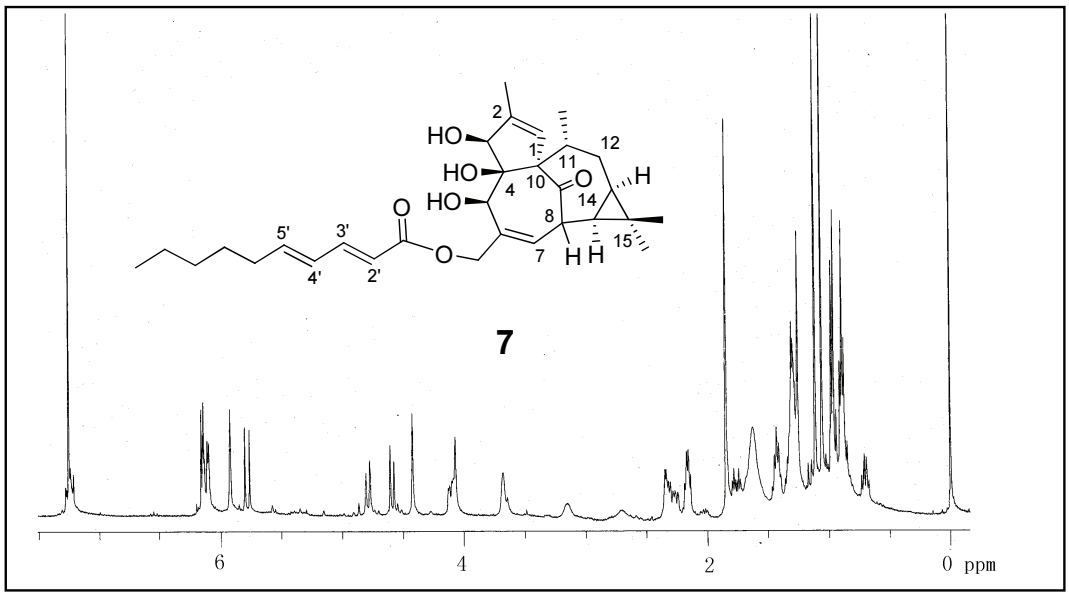



Fig. 4 $^1$H-NMR($CDCl_3$) of compound **7**

$^{13}$C-NMR and $^{1}$H-NMR of compound **7** shown a different pattern from that of an *E,Z*- or *Z,E*-configuration in the double bonds of the ester residue. In the case of **7**, H-2′ appeared at δ 5.78 as a doublet ($J$2′,3′ = 15.2 Hz), corresponding to a 2′,3′-trans double bond. The overlapping signals at δ 6.14, attributed to the H-4′, H-5′ signals, did not offer much information as to the nature of the double bond. But all the signals marched well with that of reported datas which confirmed to be a *E,E*-configuration in the double bonds of the ester residue. Thus far the structure of **7** was established as 20-*O*-(2′*E*,4′*E*-decadienoyl)ingenol.

It is reported that Euphol (**1**) together with some other this type of triterpenes [5b] show a moderate cytotoxicity on fibroblasts, $ID_{50}$ about 100 μg/mL. Wang et al [8] reported that the isolation of compounds **5**, **6**, and **7** by a bioassay guided method and these diterpenes shown potent anticancer activity against *xenopus* cell line at concentration of 0.5 μg/mL. There is no bioactivity data of compound **8** reported.

With these compounds in hand, our next step is to assay their cytotoxicity in vitro and scale up those compounds with potent activity for bioassay in vivo. At the same time identification of detoxicants of the water extract of ZJ will be carried.

## 3. Experimental Section

**3.1 General Experimental Procedures.** NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are expressed in $\delta$ values. FABMS and HRFABMS measurements were carried out on a JEOL JMS-700T spectrometer, and glycerol was used as a matrix. Column chromatography was performed with normal-phase silica gel (silica gel 60N, spherical, neutral, 40-50 $\mu$m, Kanto Chemical Co., Inc.) and reversed-phase silica gel (Cosmosil 75C18-OPN, Nacalai Tesque Inc.). Medium-pressure liquid chromatography (MPLC) was performed with a Buchi pump module C-650 system. Preparative TLC was carried out on precoated silica gel 60F254 and RP-18F254 plates (Merck, 0.25 or 0.50 mm thickness).

**3.2 Plant Material.** The dried roots of *Euphorbia kansui* L. were collected in Xianyang, Sannxi Province, People's Republic of China, in October 1999. A voucher specimen was deposited at the Museum of Materia Medica, Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Japan.

**3.3 Extraction and Isolation.** The dried roots of *E. kansui* (1 kg) were extracted twice with water under reflux. Evaporation of the solvent under reduced pressure from the combined aqueous solution gave the aqueous extract (200 mL). The extract was extraced with ethyl acetate (3 X 200 mL). The amount extracted was 10 g, and the residual aqueous extract weighed 23 g.

The ethyl acetate fraction was subjected to silica gel column chromatography with MPLC using an EtOAc-hexane gradient system. The column chromatographic fractions (100 mL each) were combined according to TLC monitoring into 10 subfractions (Fig. 5).

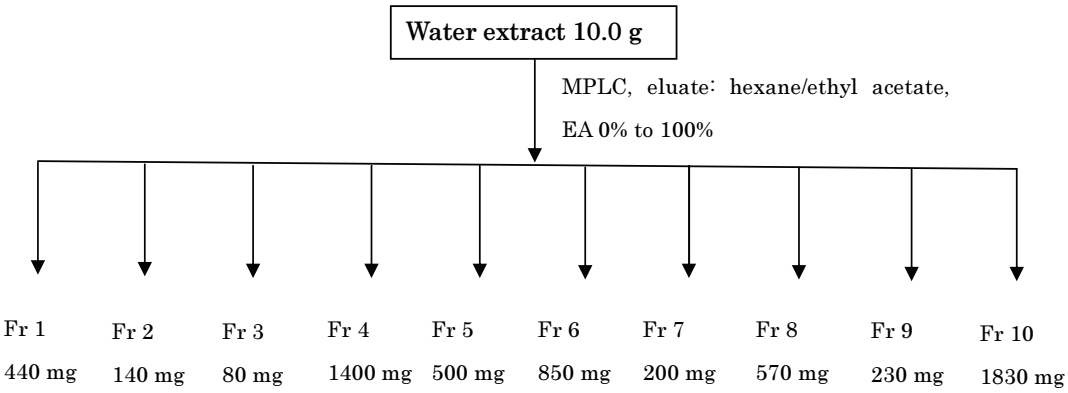



Fig. 5

Fr **1** and **2** (580 mg): Combined Fr **1** and **2** was rechromatographed on silica gel with MPLC using a chloroform-hexane gradient system to afford 32 subfractions. NMR ($^{1}H$, $^{13}C$, $CDCl_3$) indicated that the main component was fatty acid esters and wax.

Fr **4** (1400 mg): Fr **4** was rechromatographed on silica gel with MPLC using a EtOAc-hexane gradient system to afford a white crystal (**1**, 952 mg) as the main component.

Fr **5** and **6** (1350 mg): Combined Fr **5** and **6** was rechromatographed on silica gel with MPLC using a chloroform-hexane gradient system to afford 25 subfractions. NMR ($^{1}H$, $^{13}C$, $CDCl_3$) indicated that the main component was fatty acids.

Fr **7** (200 mg): Fr 7 was subjected to normal-phase preparative TLC (NPTLC) with Hexane/ $CHCl_3$/EtOAc (5:12:1) afford 7 subfractions (Fig. 6). Further separation of Fr **7-4** (30 mg) by semipreparitve HPLC (Waters Discovery C18 column, 10 X 250 mm, 5 μM, MeOH-$H_2O$, 96:4, flow rate 2 mL/min; UV detector set at 211 or 254 nm) based on TLC, afford **7-4-1** (1.4 mg, Rt 10.9 min) and **7-4-2** (2.4 mg, Rt 12.3 min), NMR ($^{1}H$, $^{13}C$, $CDCl_3$) confirmed that **7-4-2** was a mixture of two ingenol-typed diterpene esters.

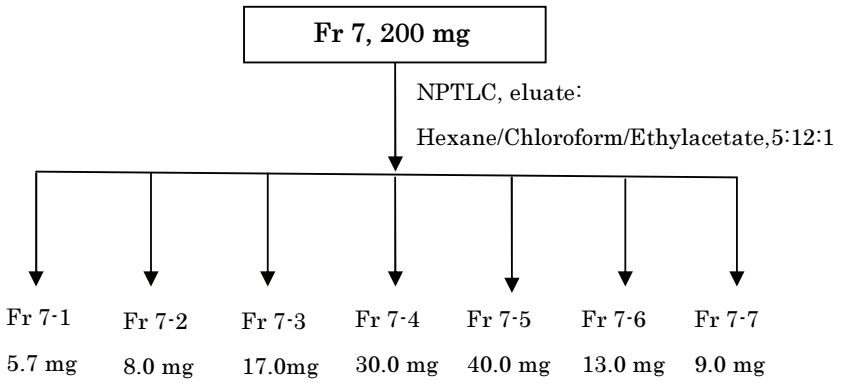



Fig. 6

Fr **8** (570 mg): Fr **8** was subjected to normal-phase preparative TLC with MeOH/$CHCl_3$/ (1:9) afford 8 subfractions (Fig. 7). Further separation of Fr **8-0** (110 mg) by semipreparitve HPLC (Waters Discovery C18 column, 10 X 250 mm, 5 μM, MeOH-$H_2O$, 88:12, flow rate 2 mL/min; UV

detector set at 211 or 254 nm) based on TLC, afford Fr **8-0-1** (5.4 mg, Rt 9.5 min) and **8-0-2** (5.4 mg, Rt 11.2 min), but NMR ($^1$H, $CDCl_3$) confirmed that each of them is not pure compound, there contain two isomers. Combined Fr **8-0-1** and **8-0-2** further purified by NPTLC (hexane/EA, 4:1), furnished compounds **8-0-2-1** (**2**, 3.3 mg) and **8-0-2-2** (**3**, 3.3 mg), which were characterized by NMR ($^1$H, $^{13}$C, and $^1$H-COSY, $CDCl_3$).

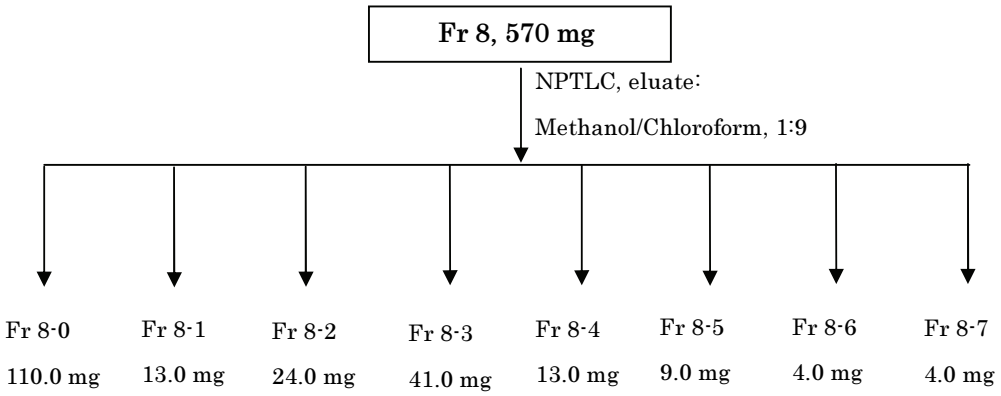


Fig. 7

Fr **9** (230 mg): Separation of Fr **9** by semipreparitve HPLC (Waters Discovery C18 column, 10 X 250 mm, 5 $\mu$ M, MeOH-$H_2O$, 92:8, flow rate 2 mL/min; UV detector set at 211 or 254 nm) afford **9-1** (< 0.2 mg, Rt 3.2 min), **9-2** (1 mg, Rt 7.9 min), **9-3** (5.5 mg, Rt 10.0 min), **9-4** (**4**, 8.7 mg, Rt 12.9 min), and **9-5** (**5**, 4.0 mg, Rt 13.8 min), which were characterized by NMR ($^1$H, $^{13}$C, $CDCl_3$) and compared with Ref.

Fr **10** (900 mg): Fr **10** was rechromatographed on reverse-phase silica gel with MPLC using a acetone-water gradient system to afford 8 subfractions (Fig. 8) based on TLC. Further separation of Fr **10-4** (120 mg) by semipreparitve HPLC (Waters Discovery C18 column, 10 X 250 mm, 5 μM, MeOH-$H_2O$, 85:15, flow rate 2 mL/min; UV detector set at 211 or 254 nm) based on TLC, afford **10-4-1** (4.1 mg, Rt 13.5 min), **10-4-2** (**6**, 10 mg, Rt 17.5 min), **10-4-3** (9.0 mg, Rt 19.0 min), **10-4-4** (11.7 mg, Rt 22.5 min), **10-4-5** (2.7 mg, Rt 25.5 min). NMR ($^1$H, $^{13}$C, $CDCl_3$) confirmed that except **10-4-2** was pure other four fractions were mixtures of ingenol-typed diterpene. Further purified **10-4-3** by NPTLC ($CHCl_3$/EA, 5:1) furnished **10-4-3-1** (**7**, 3.5 mg). Further purified **10-4-4** by NPTLC ($CHCl_3$/EA, 5:1) furnished **10-4-4-1** (1.4 mg), **10-4-4-2** (**8**, 1.5 mg), **10-4-4-3** (1.4 mg).

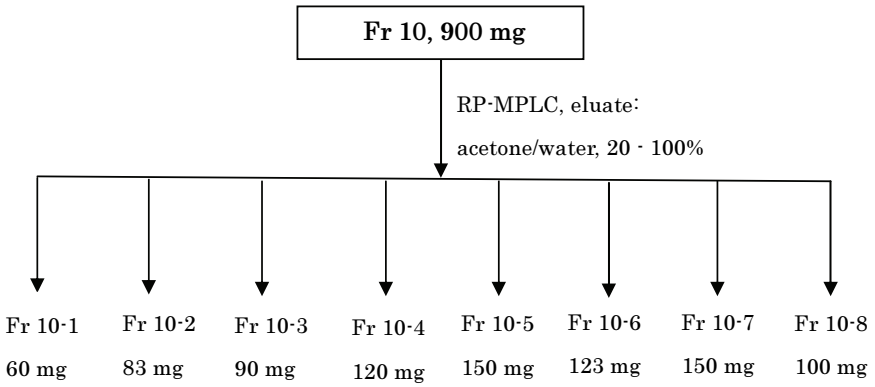


Fig. 8

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