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Quantitative Determination of Triterpenoids from the Fruits of *Zizyphus jujuba*

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Abstract – Eleven triterpenoids, colubrinic acid (**1**), alphitolic acid (**2**), 3-*O-cis-p*-coumaroyl alphitolic acid (**3**), 3-*O-trans-p*-coumaroyl alphitolic acid (**4**), 3-*O-cis-p*-coumaroyl maslinic acid (**5**), 3-*O-trans-p*-coumaroyl maslinic acid (**6**), betulinic acid (**7**), oleanolic acid (**8**), betulonic acid (**9**), oleanonic acid (**10**), and zizyberenalic acid (**11**), were isolated from the fruits of *Zizyphus jujuba* Mill. A simple and rapid HPLC method, using a C_{18} column, has been developed for the quantitative analysis of these compounds **1** (0.74%), **2** (0.09%), **3** (0.19%), **4** (0.19%), **5** (0.08%), **6** (0.08%), **7** (0.41%), **8** (0.05%), **9** (0.50%), **10** (0.59%), and **11** (0.19%).
Keywords – *Zizyphus jujuba*, triterpenoid, quantitative analysis

Introduction

Zizyphus jujuba is a thorny rhamnaceous plant widely distributed in Europe and Southeastern Asia. The seed, known as Suan Tsao Zen in China, is a famous Chinese medicine used for the treatment of insomnia (Namba, 1993). Until recently, several triterpenes ([a]Yagi *et al.*, 1978; [b]Yagi *et al.*, 1978; Lee *et al.*, 1996) and cyclopeptide alkaloids (Tripathi *et al.*, 2001) have been isolated from the seed, fruit, and root of this plant. In the previous paper, we reported the isolation and cytotoxic activity of the triterpenoic acids, colubrinic acid (**1**), alphitolic acid (**2**), 3-*O-cis-p*-coumaroyl alphitolic acid (**3**), 3-*O-trans-p*-coumaroyl alphitolic acid (**4**), 3-*O-cis-p*-coumaroyl maslinic acid (**5**), 3-*O-trans-p*-coumaroyl maslinic acid (**6**), betulinic acid (**7**), oleanolic acid (**8**), betulonic acid (**9**), oleanonic acid (**10**), and zizyberenalic acid (**11**), from this plant source. It had been reported that, 3-*O-cis*, and *trans-p*-coumaroyl alphitolic acids (**3**, **4**) showed significant cytotoxic activities against K562, B16 (F-10), SK-MEL-2, PC-3, LOX-IMVI and A549 tumor cell lines (Lee *et al.*, 2003). Herein we present the quantitative analysis of eleven triterpenoids from fruit of *Z. jujuba* by simple HPLC method.

Exprimental

General – The chromatographic system for quantitative analysis consisted of a L-7100 pump (Hitachi, Japan), L-7400 UV detector (Hitachi, Japan), D-2500 Chromato-Integrator (Hitachi, Japan) and Rheodyne 7725 injector with a 20 μl sample loop. Separations were performed using an YMC-Pack (YMC Co., Ltd., Kyoto, Japan) C_{18} column (150×4.6 mm i. d. 5 μm). Methanol (Burdick & Jackson, USA), acetonitrile (Burdick & Jackson, USA) used in this work were of HPLC grade and other reagents were of analytical grade. Milli-Q (Millipore, MA, USA) treated water (with resistivity more than 18 MΩ cm) was used throughout the experiment.

Plant material – Fruit of *Zizyphus jujuba* was purchased from an oriental medicine markets in Kumsan, Korea. A voucher specimen (KT&G 0005) was deposited in the herbarium of the KT&G Central Research Institute.

Preparation of triterpenoid standards – Eleven triterpenoic acids were isolated from Zizyphi Fructus as reported previously (Lee *et al.*, 2003) and identified by ^{1}H-NMR, ^{13}C-NMR and ESIMS. The purity confirmed by HPLC was more than 99.5%.

Preparation of test sample – Air-dried fruits (10 g) was finely powdered and percolated with 100 mL MeOH at room temperature (27°C) for 48 h, and filtered using filter paper. The MeOH solution filtered using a Waters sample filtration Kit ("Organic"), and a 10 μL sample subjected to HPLC analysis.

HPLC analysis – The HPLC separation of eleven triterpenoids for quantitative analysis was performed using a reverse phase system (YMC-Pack, C_{18}, 150×4.6 mm i. d. 5 μm). Elution was initially with acetonitrile-water (50 -50), which was changed according to linear gradient over 30 min to acetonitrile-water (100-0). The flow rate was 1

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Table 1. The calibration functions and contents of the components in Zizyphi Fructus

Compound[a]	Rt (min)	Linear regression equation[b,c]	r-Value	Content (%)[d]
1	14.67	y = 230399 x + 1102	0.99998	0.74 ± 0.13
2	16.66	y = 107484 x − 3728	0.99883	0.09 ± 0.02
3	19.82	y = 1271930 x − 1210	0.99991	0.19 ± 0.06
4	20.42	y = 1281864 x + 2339	0.99991	0.19 ± 0.02
5	21.26	y = 1498194 x + 1520	0.99956	0.08 ± 0.02
6	22.39	y = 1590801 x + 1304	0.99988	0.08 ± 0.01
7	22.87	y = 230320 x − 3210	0.99993	0.41 ± 0.15
8	24.36	y = 604321 x + 1010	0.99789	0.05 ± 0.06
9	26.90	y = 291012 x + 4011	0.99999	0.50 ± 0.05
10	27.76	y = 171890 x + 1101	0.99990	0.59 ± 0.04
11	29.08	y = 502811 x − 2043	0.99997	0.19 ± 0.06

[a]**1**: colubrinic acid, **2**: alphitolic acid, **3**: 3-*O-cis-p*-coumaroyl alphitolic acid, **4**: 3-*O-trans-p*-coumaroyl alphitolic acid, **5**: 3-*O-cis-p*-coumaroyl maslinic acid, **6**: 3-*O-trans-p*-coumaroyl maslinic acid, **7**: betulinic acid, **8**: oleanolic acid, **9**: betulonic acid, **10**: oleanonic acid, **11**: zizyberenalic acid.

[b]The calibration functions of each standard were obtained from the separate injections of four different concentration of standard (0.05, 0.1, 0.5, and 1.0 mg/mL).

[c]The calibration function of each constituent standard calculated with peak area (y) and concentration (x, mg/ml).

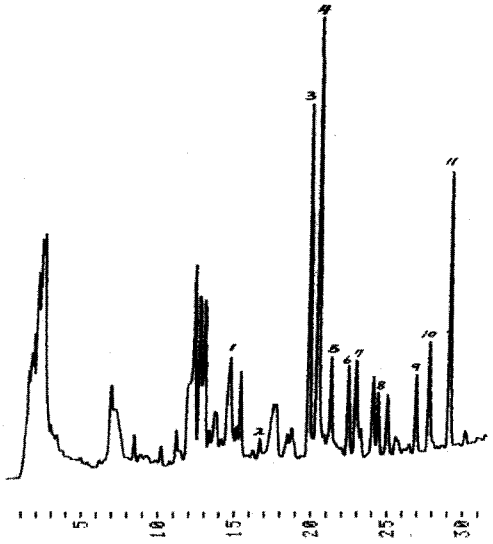
[d]Mean values (n = 3) ± standard deviation.

mL/min, and 10 μL aliquots of samples were injected for analysis and UV detection was carried out at 220 nm.

Calibration – Stock solutions (1 mg/mL) of isolated standard compounds **1-11** were prepared individually in methanol, and different concentrations (0.05, 0.1, 0.5 and 1.0 mg/ml) of these were loaded onto an HPLC for the preparation of the calibration functions (Table 1). The calibration function of each constituent standard calculated with peak area (y), concentration (x, mg/ml), and mean values (n = 3) ± standard deviation.

Result and Discussion

To select an optimal mobile phase composition for the analysis of eleven triterpenoid from MeOH extracts of the fruits of *Z. jujuba*, several HPLC runs with various concentrations of acetonitrile in water as mobile phase were performed. A solution of initial 50% acetonitrile in water, which was changed according to linear gradient over 30 min to 100% acetonitrile was selected for mobile phase. The chromatographic system employed produced symmetrical peaks with baseline resolution for all tested triterpenoids using a simple gradient profile. As shown in Fig. 1, no interference from endogenous substances was observed in any of the samples analysed. The retention time (Rt) for the eleven triterpenoids **1-11** were 14.6, 16.7, 19.8, 20.4, 21.3, 22.4, 22.9, 24.4, 26.9, 27.8, and 29.1 min, respectively. Table I shows the precision and accuracy of this method. In conclusion, an efficient procedure for the isolation and determination of 3-*O-cis-*, *trans-p*-coumaroyl alphitolic acid (**3**, 0.19%, **4**, 0.19%) and other triterpenoids have been established and the protocol could be used for the quality control of phytopreparations containing this important secondary metabolite in the herb material.



1: colubrinic acid
2: alphitolic acid
3: 3-O-cis-p-coumaroyl alphitolic acid
4: 3-O-trans-p-coumaroyl alphitolic acid
5: 3-O-cis-p-coumaroyl maslinic acid
6: 3-O-trans-p-coumaroyl maslinic acid
7: betulinic acid
8: oleanolic acid
9: betulonic acid
10: oleanonic acid
11: zizyberenalic acid.

Fig. 1. Typical reversed-phase HPLC chromatogram (detected at 220 nm) of a MeOH extract of the fruits of *Zizyphus jujuba* (for extraction and chromatographic protocols see Experimental section).

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