

Analysis of Bioactive Triterpenes in *Eriobotrya japonica* LINDL. by High-Performance Liquid Chromatography

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ABSTRACT

Eriobotrya japonica (Rosaceae) has been used for clearing away heat in the lung to stop cough and lowering the adverse flow of gi to stop vomiting. High-performance liquid chromatographic method was applied to assay the five triterpenes contents in *E. japonica* from various locations. Samples were analyzed on a HyPURITY C-18 column (5 μ m, 4.6 \times 250 mm) eluted at a rate of 0.5 mL/min with a solvent of A - B (A, Methanol; B, 0.15% acetic acid aqueous; A: B = 85: 15, v/v). Triterpenes were detected by refractive index detection (RID). Regression equations of the calibration curves revealed good linear relationships (correlation coefficients: 0.9998-1.0000). The recoveries ranged between 98.39% - 102.41% and indicated good accuracy.

Key words: *Eriobotrya japonica*, HPLC, tormentic acid, maslinic acid, corosolic acid, oleanolic acid, ursolic acid

INTRODUCTION

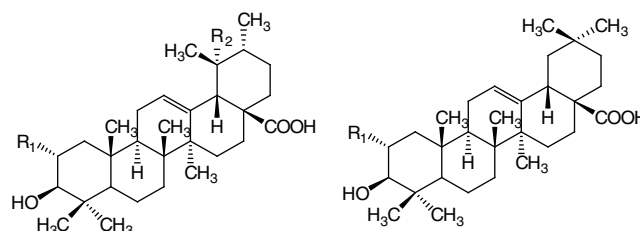
Eriobotrya japonica Lindl is a Rosaceous evergreen tree distributed in southeastern China and Taiwan. They have been used in traditional Chinese medicine for clearing away heat in the lung. It stops the cough and lowers the adverse flow of gi to stop vomiting. The five main bioactive constituents of the leaf were characterized spectroscopically as tormentic acid, maslinic acid, corosolic acid, oleanolic acid and ursolic acid (Figure1). These isolated triterpenoid compounds show several biological and pharmacological activities such as anti-oxidative⁽¹⁾, anti-inflammatory⁽²⁾, hepato-protective effects⁽³⁾, anti-tumor⁽⁴⁾, and anti-diabetes effects⁽⁵⁻⁷⁾. This paper describes the development of a simple and accurate analytical method to detect the bioactive triterpenes for the quality control of *E. japonica*. By HPLC, the contents of these five constituents in the leaves of the plant collected from different areas of Taiwan, were also determined.

EXPERIMENT

I. Materials and Reagents

Nine samples of *E. japonica* were provided by

Lecturer Chen-I Chen (Department of Bio-industry and Agribusiness Administration) in Taiwan and was harvested in September 2007 from Hualien (001,002), Taoyuan (003), Miaoli(004), Taichung(005), Nantou (006,007), Changhua (008), Taitung (009). The sample noted as 010 was purchased from herbal store in Mainland China. All materials were sorted and identified by Associate Professor Chao-Lin Kuo with voucher specimens (CMU-EJ08001-CMU-EJ08010) deposited in the China Medical University, Taichung, Taiwan. Methanol and glacial acetic acid (LC grade) were purchased from E. Merck, in addition to other reagents of analytical grade. Milli-Q plus water (Millipore, Bedford, MA, U.S.A.) was used throughout the study.



	R ₁	R ₂		R ₁
tormentic acid	OH	OH	maslinic acid	OH
corosolic acid	OH	H	oleanolic acid	H
ursolic acid	H	H		

Figure 1. Structure of the triterpenes in *E. japonica*.

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II. Purification of the Five Standard Markers

The leaf of *E. japonica* (1 kg) was purchased from herbal store and extracted with ethanol twice. The filtrate was concentrated under reduced pressure to yield a dark ethanol extract (syrup, 137.4 g), which was resuspended in H₂O, then partitioned with ethyl acetate to afford the deep green syrup (28.5 g). The ethyl acetate extract (8.0 g) was chromatographed on a silica gel (E. Merck, 70-230 mesh) using hexane-ethyl acetate (100:0-0:100) as eluting system to give ten fractions. The fraction V (513mg) was shown the same R_f value (R_f value = 0.08, MeOH/H₂O, 85 : 15) on TLC plate (E. Merck RP-18F₂₅₄S) and then further purified by preparative high performance liquid chromatography (PHPLC, column: YMC, J'Sphere series ODS-H80, 10 × 250 mm, 85% MeOH (v/v), 3 mL/min), to yield oleanolic acid (15.5 mg) and ursolic acid (20.9 mg). Fraction VIII (690 mg) was also shown the same R_f value (R_f value = 0.19, MeOH/H₂O, 85 : 15) on TLC plate and purified by PHPLC to afford maslinic acid (12.2 mg) and corosolic acid (14.9 mg). Fraction VII (452 mg, R_f value = 0.32) was purified by the same PHPLC system to get tormentic acid (25.6 mg) successfully. Five compounds were identified as tormentic acid, maslinic acid, corosolic acid⁽⁸⁾, oleanolic acid⁽⁹⁾ and ursolic acid⁽¹⁰⁾, respectively, by comparing with those spectral data in the literature.

III. Apparatus and Conditions

The HPLC was performed on a Shimadzu 10A system equipped with one pump (LC-10AT Shimadzu Japan) and a RI spectrophotometric detector (RID - 10A, Shimadzu, Japan). The HyPURITY C-18 column (5 μ m, 4.6 × 250 mm) was eluted at a rate of 0.5 mL/min with a solvent of A-B (A, Methanol; B, 0.15% Acetic acid aqueous; A : B = 85 : 15, v/v). Between each sample injection and the last run, the system was reconditioned for another 40 min.

IV. Preparation of Standard Solution

To prepare standard solutions, tormentic acid, maslinic acid, corosolic acid, oleanolic acid and ursolic acid were accurately weighed and dissolved in 85% methanol. The various concentrations were 25, 62.5, 125, 250, 500 and 750 μ g/mL for each of tormentic acid, maslinic acid, corosolic acid, oleanolic acid and ursolic acid.

V. Preparation of Sample Solution

All crude drugs were individually cut into pieces and dried at 80°C for 24 hours. Market sample (10 g) was accurately weighed and extracted with 100 mL ethanol twice. After extraction, the mixture was concentrated and the ethanol extract was resuspended in H₂O and partitioned five times with ethyl acetate. The ethyl acetate extracts were combined and dried. The sample

was then accurately weighed and dissolved in 85% methanol in a volumetric flask. This sample solution was filtered through a 0.45 μ m filter (Millipore) before use. Other samples were also processed as mentioned above.

VI. Recovery Studies

The recovery was used to evaluate the accuracy. A series of various concentrations of tormentic acid (125, 250, 500 μ g/mL), maslinic acid (25, 62.5, 125 μ g/mL), corosolic acid (62.5, 125, 250 μ g/mL), oleanolic acid (25, 62.5, 125 μ g/mL) and ursolic acid (125, 250, 500 μ g/mL) were added to the sample solution of *E. japonica* for which the contents were measured. All sample solutions were filtered, subjected to HPLC analysis. The recovery (%) was calculated by equation of $[(A - B) / C] \times 100\%$, where A is the amount measured, B is the amount of sample without standards and C is the spiked amount of the standards.

RESULTS AND DISCUSSION

I. Analytical Conditions

All five triterpenes were successfully determined by HPLC (Figure 2A). Tormentic acid, maslinic acid, coro-

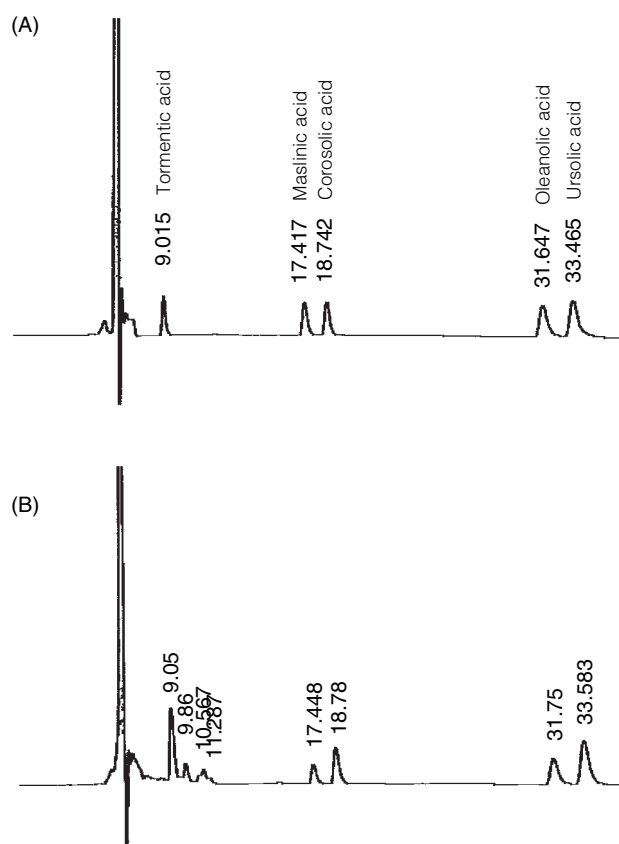


Figure 2. Chromatograms of five triterpenes (A) and market sample (B).

solic acid, oleanolic acid and ursolic acid were resolved and eluted at 9.02 min, 17.42 min, 18.74min, 31.65min and 33.46 min, respectively. Market sample (010) of analysis was determined within 40 minutes (Figure, 2B).

II. Calibration Curve for Five Triterpenes

The linearity of the plot of peak area (X) vs. concentration (Y, $\mu\text{g/mL}$) for each of the triterpene was investigated. The regression (Table 1) revealed good linear relationships (correlation coefficients: 0.9998-1.0000) between the peak areas and concentrations.

III. Reproducibility

The contents of five triterpenes were extracted and analyzed in duplicate from Market sample (010). Consequently, extract prepared as described in the section V was followed in quintuplicate. The results clearly indicated that the method proposed above had satisfactory reproducibility (Table 2).

VI. Precision

Standards of tormentic acid (125, 250, 500 $\mu\text{g/mL}$), maslinic acid (25, 62.5, 125 $\mu\text{g/mL}$), corosolic acid (62.5, 125, 250 $\mu\text{g/mL}$), oleanolic acid (25, 62.5, 125 $\mu\text{g/mL}$) and ursolic acid (125, 250, 500 $\mu\text{g/mL}$) were injected both at intra-day (injection each concentration

five times within one day) and inter-day (injection each concentration four times during a week) intervals to check

Table 1. Calibration curves of five triterpenes

Compound	Linear range ($\mu\text{g/mL}$)	Linear equations	r
tormentic acid	25-750	$y = 3507.8x + 144.07$	0.9999
maslinic acid	25-750	$y = 3920.0x + 171.35$	1.0000
corosolic acid	25-750	$y = 4765.4x + 42.93$	0.9998
oleanolic acid	25-750	$y = 5327.3x + 50.87$	1.0000
ursolic acid	25-750	$y = 5337.1x - 2.210$	0.9999

Table 2. Reproducibility of five triterpenes extracted from *E. japonica*

Compound	Content (%)
tormentic acid	0.52 ± 0.02
maslinic acid	0.14 ± 0.01
corosolic acid	0.23 ± 0.03
oleanolic acid	0.09 ± 0.01
ursolic acid	0.45 ± 0.00

(mean \pm S.D., n = 5)

Table 3. Reproducibilities of intra-day and inter-day analysis of *E. japonica*

Compound	Concentration ($\mu\text{g/mL}$)	Mean \pm S.D. (RSD %)	
		intra-day (n = 5)	inter-day (n = 4)
tormentic acid	125	124.09 ± 2.95 (2.37)	124.74 ± 2.03 (1.63)
	250	252.55 ± 2.66 (1.05)	251.86 ± 2.76 (1.10)
	500	500.93 ± 2.55 (0.51)	500.26 ± 2.70 (0.54)
maslinic acid	25	22.66 ± 0.55 (2.43)	23.31 ± 0.40 (2.99)
	62.5	63.53 ± 1.02 (1.60)	63.56 ± 0.99 (1.56)
	125	126.80 ± 2.97 (2.34)	126.30 ± 1.78 (1.41)
corosolic acid	62.5	63.58 ± 1.92 (3.02)	63.38 ± 2.10 (3.31)
	125	126.45 ± 1.63 (1.29)	123.92 ± 2.90 (0.99)
	250	252.42 ± 3.10 (1.23)	250.71 ± 2.47 (0.99)
oleanolic acid	25	25.56 ± 0.72 (2.86)	25.51 ± 0.83 (3.27)
	62.5	63.36 ± 1.29 (2.03)	63.62 ± 0.98 (1.55)
	125	124.59 ± 2.57 (2.06)	124.20 ± 1.47 (1.19)
ursolic acid	125	125.60 ± 2.97 (2.37)	124.47 ± 2.27 (1.83)
	250	249.87 ± 2.88 (1.15)	249.37 ± 1.97 (0.79)
	500	501.09 ± 2.00 (0.60)	500.78 ± 1.79 (0.36)

the reproducibility. The results (Table 3) showed that the relative standard deviation values of intra-day and inter-day were 0.51-3.02% and 0.36-3.31%, respectively, suggesting that both of them had good precision.

V. Recovery

The results were shown in Table 4 and the recoveries of five triterpenes ranged from 98.39 to 100.62% for

Table 4. Recovery of five triterpenes in *E. japonica* (n = 5)

Compound	Concentration (µg/mL)	Recovery (%) Mean ± S.D. (RSD %)
tormentic acid	125	98.39 ± 2.65 (2.69)
	250	100.62 ± 1.88 (1.87)
	500	99.53 ± 2.30 (2.31)
maslinic acid	25	100.47 ± 2.30 (2.30)
	62.5	99.63 ± 2.07 (2.09)
	125	101.61 ± 2.38 (2.34)
corosolic acid	62.5	100.78 ± 1.13 (1.12)
	125	100.65 ± 1.16 (1.15)
	250	102.41 ± 3.92 (3.93)
oleanolic acid	25	101.36 ± 0.73 (0.73)
	62.5	98.63 ± 2.46 (2.50)
	125	100.63 ± 1.33 (1.32)
ursolic acid	125	101.87 ± 2.00 (1.96)
	250	100.11 ± 2.23 (2.23)
	500	101.79 ± 1.62 (1.59)

tormentic acid, 99.63 to 101.61% for maslinic acid, 100.65 to 102.41% for corosolic acid, 98.63 to 101.36% for oleanolic acid and 100.11 to 101.87% for ursolic acid, respectively.

VI. Quantitative Determination

In order to evaluate the locations of *E. japonica*, the contents of ten samples were determined by HPLC. The result was summarized in Table 5 and it showed great variations among the contents of tormentic acid, ursolic acid and corosolic acid in *E. japonica* from different locations.

In this study, we established a simple and accurate method for the simultaneous determination *E. japonica* from various locations. Five triterpenes including tormentic acid, maslinic acid, corosolic acid, oleanolic acid and ursolic acid were selected as markers for the quality control of crude drugs containing *E. japonica* in the future.

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Table 5. The contents of five triterpenes in ten samples of *E. japonica* from different locations

Batch	location	Compound (%)				
		tormentic acid	maslinic acid	corosolic acid	oleanolic acid	ursolic acid
001	Hualien	0.37 ± 0.00	0.12 ± 0.00	0.34 ± 0.00	0.10 ± 0.01	0.54 ± 0.01
002	Hualien	0.18 ± 0.01	0.10 ± 0.01	0.18 ± 0.00	0.07 ± 0.00	0.35 ± 0.01
003	Taoyuan	0.57 ± 0.02	0.15 ± 0.03	0.28 ± 0.01	0.10 ± 0.01	0.47 ± 0.03
004	Miaoli	0.29 ± 0.02	0.10 ± 0.00	0.13 ± 0.00	0.07 ± 0.00	0.39 ± 0.01
005	Taichung	0.38 ± 0.01	0.10 ± 0.01	0.12 ± 0.00	0.05 ± 0.00	0.26 ± 0.01
006	Nantou	0.36 ± 0.01	0.12 ± 0.01	0.17 ± 0.00	0.06 ± 0.00	0.41 ± 0.01
007	Nantou	0.33 ± 0.01	0.09 ± 0.01	0.31 ± 0.01	0.12 ± 0.02	0.67 ± 0.01
008	Changhua	0.23 ± 0.01	0.06 ± 0.01	0.18 ± 0.01	0.06 ± 0.00	0.39 ± 0.02
009	Taitung	0.27 ± 0.02	0.09 ± 0.01	0.25 ± 0.01	0.06 ± 0.00	0.34 ± 0.01
010	Market	0.52 ± 0.01	0.13 ± 0.01	0.24 ± 0.01	0.10 ± 0.01	0.45 ± 0.01

(mean ± S.D., n = 3)

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