Journal of Food and Drug Analysis

Volume 16 | Issue 6 Article 5

2008

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*Journal of Food and Drug Analysis, Vol. 16, No. 6, 2008, Pages 41-45* 藥物食品分析 第十六卷 第六期

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

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(Received: March 26, 2008; Accepted: September 9, 2008)

## 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 μm, 4.6 × 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

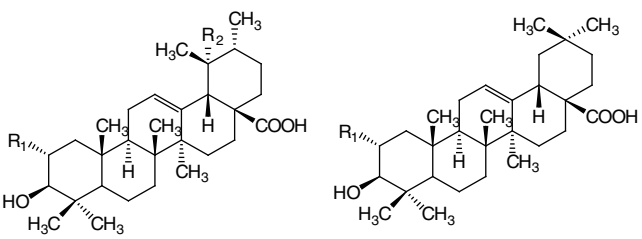
## INTRODUCTION

*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.




| | $R_1$ | $R_2$ |
|---|---|---|
| tormentic acid | OH | OH |
| corosolic acid | OH | H |
| ursolic acid | H | H |

| | $R_1$ |
|---|---|
| maslinic acid | OH |
| oleanolic acid | 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 $H_2O$, then partitioned with ethyl acetate to afford the deep green syrup (28.5 g). The ethyl acetate extract (8.0 g) was chromatographied 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/$H_2O$, 85 : 15) on TLC plate (E. Merck RP-18$F_{254S}$) 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/$H_2O$, 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 µm, 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 µg/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 $H_2O$ 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 µm 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 concentractions of tormentic acid (125, 250, 500 µg/mL), maslinic acid (25, 62.5, 125 µg/mL), corosolic acid (62.5, 125, 250 µg/mL), oleanolic acid (25, 62.5, 125 µg/mL) and ursolic acid (125, 250, 500 µg/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] × 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 (Figrue 2A). Tormentic acid, maslinic acid, coro-

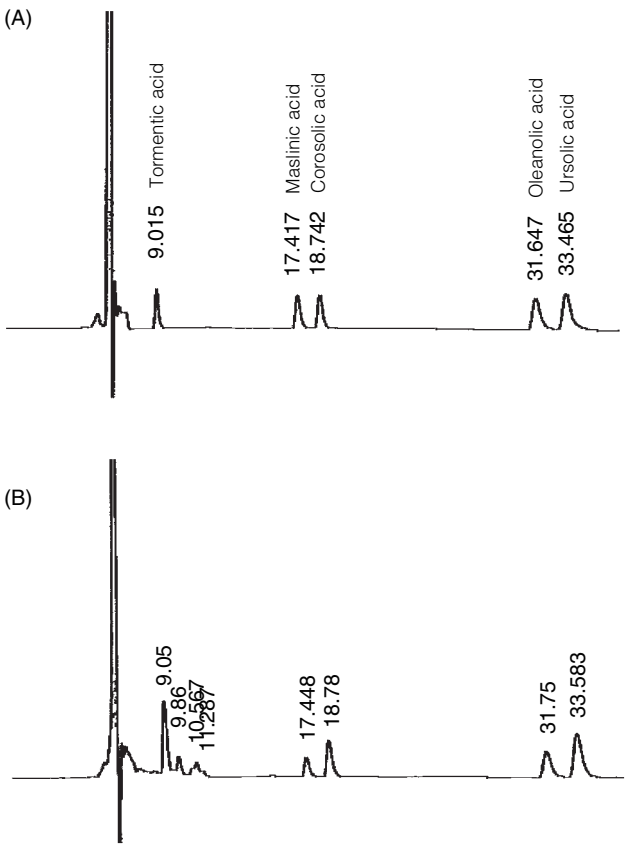


**Figure 2.** Chromatograms of five triterpenes (A) and market sample (B).

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 (Figrue, 2B).

II. *Calibration Curve for Five Triterpenes*

The linearity of the plot of peak area (X) vs. concentration (Y, μg/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 μg/mL), maslinic acid (25, 62.5, 125 μg/mL), corosolic acid (62.5, 125, 250 μg/mL), oleanolic acid (25, 62.5, 125 μg/mL) and ursolic acid (125, 250, 500 μg/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 (μg/mL) | Linear equations | r |
|---|---|---|---|
| tormentic acid | 25-750 | y = 3507.8 x + 144.07 | 0.9999 |
| maslinic acid | 25-750 | y = 3920.0 x + 171.35 | 1.0000 |
| corosolic acid | 25-750 | y = 4765.4 x + 42.93 | 0.9998 |
| oleanolic acid | 25-750 | y = 5327.3 x + 50.87 | 1.0000 |
| ursolic acid | 25-750 | y = 5337.1 x - 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 ± S.D., n = 5)

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

| Compound | Concentration (μg/mL) | Mean ± 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 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.

**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) |

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 tormantic 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.

## ACKNOWLEDGEMENTS

This study was supported by Grants to the Interchange Association (Japan) and the National Science Council. This study was also supported by grant CCMP-97-RD001 from Committee on Chinese Medicine and Pharmacy, Department of Health, Executive Yuan, Taiwan.

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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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