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Analysis of Flavonoids in *Vernonia Paltula* by High-performance Liquid Chromatography

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ABSTRACT

Lin-nan-yeh-chu, is the dried entire plant of *Vernonia paltula* (Compositae) and used as folk medicine in Taiwan. To evaluate the quality of *V. paltula*, a simple, rapid and accurate high-performance liquid chromatographic (HPLC) method was developed for the assay of four flavonoids apigenin (API), apigenin-7-*O*-glucoside (APG), luteolin (LUT) and luteolin-7-*O*-glucoside (LUG). The present HPLC system uses an Inertsil ODS-2 column by gradient elution with acetonitrile and 0.1% (v/v) phosphoric acid as the mobile phase. Ethyl paraben was used as an internal standard and detected at 254 nm. Regression equations revealed good linear relationships (correlation coefficients: 0.9998-0.9999) between the peak-area ratios of each constituent to ethyl paraben. The recovery of four marker constituents ranged from 89.3 to 95.6%. The contents of the four constituents in stem, flower, leaf and root parts of *V. paltula* have been compared. Leaf part consisted of the highest contents of flavonoids except for APG which is less than that in the flower. The root and stem only showed trace amount of APG but not the other three flavonoids. The contents of four constituents were 0.6016, 0.0042, 0.2160 and 0.0577 mg/g for apigenin-7-*O*-glucoside, apigenin, luteolin-7-*O*-glucoside and luteolin, respectively. HPLC chromatograms of another three plants of genus of *Vernonia* in Taiwan, *V. cinerea*, *V. elliptica* and *V. gratiosa*, have also been compared.

Key words: pharmaceutical analysis; *Vernonia paltula*; flavonoid; HPLC

INTRODUCTION

The consumption of folk herbs seems be growing in Taiwan, therefore the quality control for folk herbs is needed. In our laboratory, we have developed several high-performance liquid chromatography (HPLC)[(1,2)], capillary electrophoresis[(3)] and gas chromatography/mass spectrometry[(4)] methods for the analysis of marker constituents in herbal medicines.

Lin-nan-yeh-chu, also known as Hsien-hsia-hua, is the dried entire plant of *Vernonia paltula* (Compositae) and used as folk medicine in Taiwan. It has anti-inflammatory, antipyretic and anti-bacterial effects and is used to treat cold and hepatitis[(5)]. In addition to *V. paltula*, there are three other *Vernonia* plants in Taiwan[(6)]; they are *V. cinerea*, *V. elliptica* and *V. gratiosa*.

Four flavonoids: apigenin (API), apigenin-7-*O*-glucoside (APG), luteolin (LUT) and luteolin-7-*O*-glucoside (LUG), have been isolated from the plant. Flavonoids have shown many biological and pharmacological activities[(7)], and could be used as markers for chemical evaluation.

An HPLC method for the analysis of four flavonoids, API, APG, LUT and LUG, in *Ixeris laevigata var. oldhami* (Compositae) [(2)] has been reported in a previous study and applied to this experiment to achieve good results. The contents of these four constituents in different parts of *V. paltula* have been determined. Additionally, comparisons were made among HPLC chromatograms of *V. cinerea, V. elliptica and V. gratiosa.*

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EXPERIMENTAL

I. *Reagents and Materials*

APG, API, LUG and LUT were isolated from the entire plant of Vernonia paltula[(7)]. Ethyl paraben was purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from Labscan (Dublin, Ireland). Phosphoric acid was of analytical reagent grade. Ultra-pure distilled water with a resistance greater than 18 MΩ was used. Samples of *V. paltula, V. cinerea, V. elliptica* and *V. gratiosa* were obtained from Taichung and Nantou counties of Taiwan. All samples were identified by Professor Chung-Chuan Chen, Institute of Chinese Pharmaceutical Sciences, China Medical College.

II. *Apparatus and Conditions*

HPLC was performed on a Hitachi Model L-6200 Intelligent pump system equipped with a Hitachi Model L-3000 Photo Diode Array and a Shimadzu SIL-9A auto-injector. Detector was set at 254 nm. Satisfactory separation of the marker substance was obtained with reversed phase column (Inertsil ODS-2, 5 μm, 25 cm × 4.6 mm I.D.) eluted at a rate of 1.0 ml/min with a linear gradient of acetonitrile and

0.1% (v/v) phosphoric acid (Table 1). Between each sample injection and the last run, the system was reconditioned for another 20 min.

III. *Preparation of Standard Solution*

To prepare standard solutions of APG, API, LUG and LUT, an appropriate amount of internal standard solution was added to an accurately weighed standard of APG, API, LUG and LUT which were dissolved in 70 % methanol. The various concentrations were 2.6, 5.2, 10.4, 20.8 and 41.6 μg/ml for APG, 4.5, 9.0, 18.0, 36.0 and 72.0 μg/ml for API, 2.7, 5.4, 10.8, 21.6 and 43.2 μg/ml for LUG and 3.6, 7.2, 14.4, 28.8 and 57.6 μg/ml for LUT. Calibration graphs were plotted subsequently after linear regression analysis of the peak area ratios against concentrations of markers.

Table 1. HPLC elution program.

Time (min)	Acetonitrile	0.1% (v/v) Phosphoric acid
0	10	90
12	30	70
32	40	60

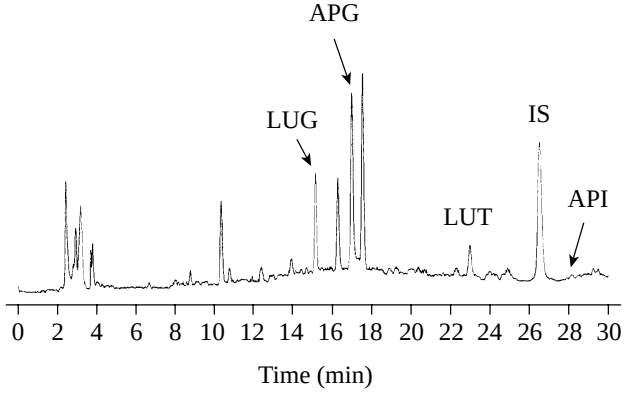

Figure1. Chromatogram of 70 % methanol extract of *V. paltula.* APG, apigenin-7-O-glucoside (60.2 μg/ml); API, apigenin (2.8 μg/ml); LUG, luteolin-7-O-glucoside (21.6 μg/ml); LUT, luteolin 5.8 (μg/ml); IS = internal standard (ethyl paraben); HPLC conditions, column: Inertsil ODS-2, 5 μm, 25 cm × 4.6 mm I.D.; mobile phase: A-B [A = acetonitrile; B = 0.1% (v/v) phosphoric acid], 0 min, 10:90; 12 min, 30:70; and 32 min, 40:60; flow rate: 1.0 ml/min; detection wavelength: 254 nm.

IV. *Preparation of Sample Solution*

Twenty grams of stem, flower, leaf and root part of *V. paltula* were individually cut to pieces and mixed well. Two grams of this sample were extracted two times (15 and 12 ml, successively) with 70% methanol by refluxing at 80˚C, one hour each time. The extracts were combined and filtered into a volumetric flask, 70% methanol was added to 25 ml. The sample solution was prepared by adding 2.5 ml of above solution and 0.5 ml of ethyl paraben solution (200 μg/ml) into a 5 ml volumetric flask and then adequate 70% methanol was added.

Two grams of the dried entire plant of *V. paltula, V. cinerea, V. elliptica* and *V. gratiosa* samples were extracted two times (15 and 12 ml, successively) with 70% methanol and then processed as above.

V. *Recovery Studies*

Three different concentrations of markers: 2.5, 5.0 and 10.0 μg/ml for APG, 1.0, 1.9 and 3.8 μg/ml for API, 2.1, 4.2 and 8.4 μg/ml for LUG and 0.5, 1.0 and 2.0 μg/ml for LUT were added to sample solution of which the marker contents

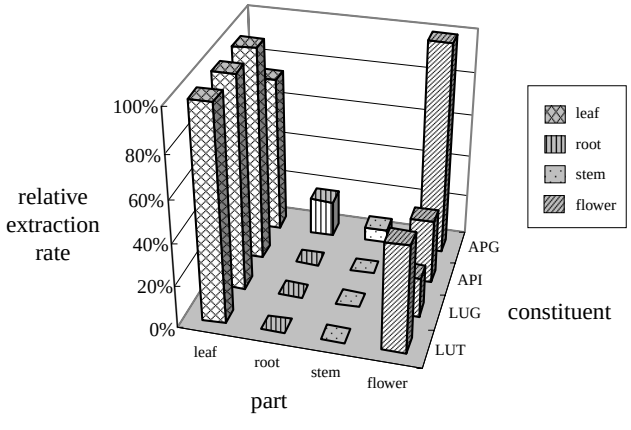

Figure 2. Comparison of contents of four constituents in different parts of *V. paltula.* For abbreviations, see Fig. 1.

Table 2. Recoveries of APG, API, LUG and LUT in *V. paltula.*

Constituent	Amount added (μg/ml)	Recovery (%)	Mean ± S.D. (%)	R.S.D. (%)
	2.5	95.5		
APG	5.0	97.1	95.6 ± 1.1	1.2
	10.0	94.3		
	1.0	84.6		
API	1.9	88.9	86.9 ± 1.8	2.0
	3.8	87.2		
	2.1	91.4		
LUG	4.2	95.6	92.4 ± 2.3	2.5
	8.4	90.3		
	0.5	90.7		
LUT	1.0	93.0	89.9 ± 3.0	3.3
	2.0	85.9		

*n=3

Journal of Food and Drug Analysis, Vol. 10, No. 3, 2002

have been determined. To each solution, a suitable amount of internal standard was added to yield a final concentration of 20.0 µg/ml. All samples were filtered through a 0.45 µg syringe filter (Gelman) and subjected to HPLC analysis.

Recovery (%) = [(Amount measured - Amount of entire plant)/Amount added)] × 100%

RESULTS AND DISCUSSION

The photodiode array detection facilitated the identification and confirmation of these four constituents. Figure 1 presents the chromatogram of *V. paltula* showing the separation of the constituents with the retention times of 26.7 min for the internal standard; 15.2 min for LUG; 17.0 min for APG; 23.1 min for LUT and 28.3 min for API. The analysis can be completed within half an hour.

Calibration graphs were constructed in the range 2.6-41.6 µg/ml for APG, 4.5-72.0 µg/ml for API, 2.7-43.2 µg/ml for LUG and 3.6-57.6 µg/ml for LUT. The regression equations of these curves and their correlation coefficients were calculated as follows: APG, y = 1.30E-02 x - 1.33E-02 (r=0.9999); API, y = 2.82E-02 x -2.73E-02 (r=0.9999); LUG, y = 1.87E-02 x - 2.00E-02 (r=0.9999) and LUT, y = 3.70E-02 x -4.70E-02 (r=0.9999). It showed good linear relationships between the peak area ratios of markers to internal standard and the concentrations of markers. A signal three times to the noise was regarded as the detection limit. The detection and quantification limits of these four constituents APG, API, LUG and LUT were 0.2, 0.6, 0.6 and 0.4 µg/mL, 0.5, 1.5, 1.5 and 1.2 µg/mL, respectively.

The results for the recoveries of APG, API, LUG and LUT were 95.6, 86.9, 92.4 and 89.9%, respectively (Table 2). The R.S.D.s of recoveries of four constituents ranged between 1.2-3.3%.

When the sample solution was analyzed by HPLC, the peaks of APG, API, LUG and LUT were confirmed by comparison of the retention time with standards. The extraction yields of the four constituents in different part of *V. paltula* were shown in Figure 2 and the highest yields were marked as 100%. Leaf part consisted of the highest contents of flavonoids except APG which is less than that in the flower. The root and stem only showed trace amount of APG but not other three flavonoids. The contents of four constituents in the entire plant of *V. paltula* were 0.6016, 0.0042, 0.2160 and 0.0577 mg/g for APG, API, LUG and LUT, respectively. The contents of APG and LUG (flavonoid glycoside) were higher than those of API and LUT (aglycone). It revealed that major flavonoids were of glycoside type. This result was similar to *Ixeris laevigata var. oldhami*[2].

Figure 3 showed the chromatograms of *V. cinerea*, *V. elliptica* and *V. gratiosa*. Since there are differences in contents of constituents among these herbs, it is suggested that identification steps should be taken before medical uses.

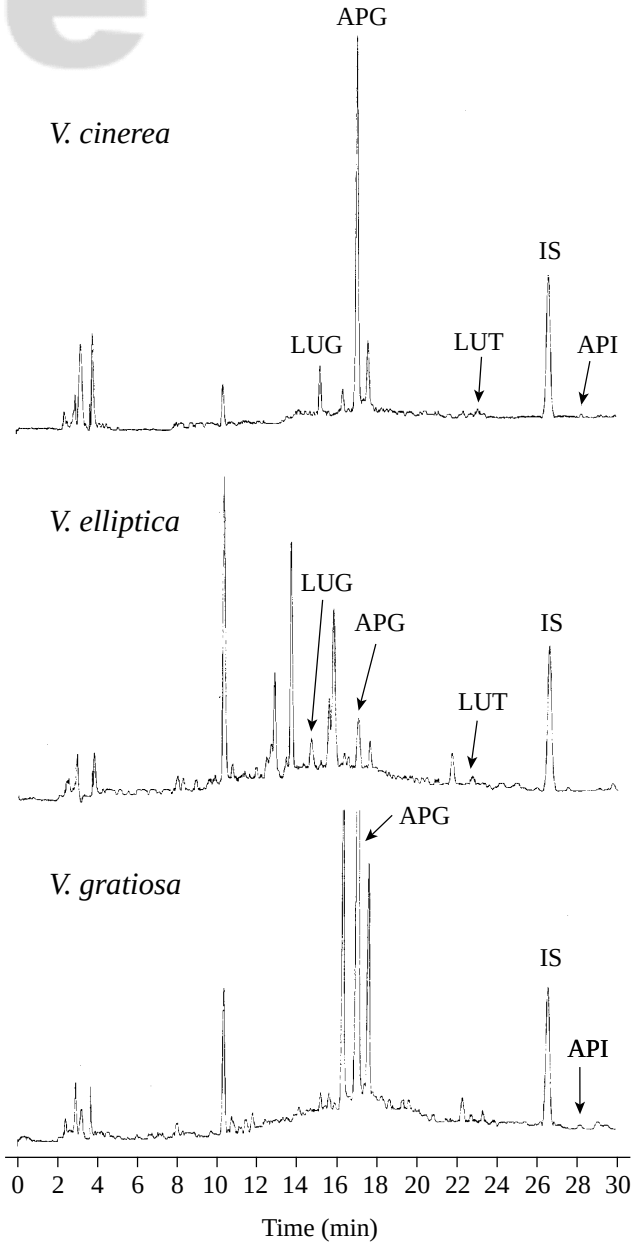

Figure 3. Chromatograms of *V. cinerea, V. elliptica and V. gratiosa,*. For HPLC conditions and abbreviations, see Fig. 1.

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高效液相層析分析嶺南野菊之黃酮類成分

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摘　要

嶺南野菊（*Vernonia paltula*）屬菊科植物，其乾燥全草是一種台灣的民間藥。本研究建立一個快速準確的高效液相層析分析方法，分析四種黃酮類成分（apigenin, apigenin-7-*O*-glucoside, luteolin 及luteolin-7-*O*-glucoside）。本實驗採用逆相層析管柱（Inertsil ODS-2），沖提液為乙腈P0.1% (v/v)磷酸水溶液，檢測波長為254 nm，內部標準品為ethylparaben作分析。四種黃酮類成分檢量線之相關係數(r)為0.9998至0.9999，均呈現良好線性關係。四種成分之回收率由89.3至95.6%，顯示準確性佳。本研究並探討四種黃酮類成分在嶺南野菊之花、葉、莖、根之含量，結果在葉部除apigenin-7-*O*-glucoside之含量低於花部外，其他三種成分之含量均最高；而根及莖部除含少量的apigenin-7-*O*-glucoside外，均不含其他成分。嶺南野菊之四種成分之含量分別為apigenin-7-*O*-glucoside 0.6016 mg/g、apigenin 0.0042 mg/g、luteolin-7-*O*-glucoside 0.2160 mg/g及luteolin 0.0577 mg/g。本研究同時採用高效液相層析方法，分析台灣另外三種*Vernonia*屬植物：一枝香（*V. cinerea*）、光耀藤（*V. elliptica*）及過山龍（*V. gratiosa*）。

關鍵詞：中藥分析，嶺南野菊，黃酮類，高效液相層析儀