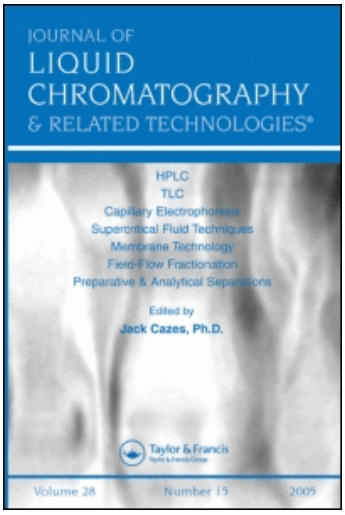
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

ANALYSIS OF FLAVONOIDS AND COUMARINS IN *IXERIS LAEVIGATA* VAR. *OLDHAMI* BY HPLC

Kuo -Liang Lu[ab]; Yoe -Ray Ku[ac]; Kuo -Ching Wen[c]; Li -Kang Ho[d]; Yuan -Shiun Chang[a]
[a] Institute of Chinese Pharmaceutical Sciences, China Medical College, Taichung, Taiwan [b] Chung Hwa Institute of Technology, Tainan Hsien, Taiwan [c] Department of Health Executive Yuan, National Laboratories of Foods and Drugs, Taipei, Taiwan [d] Department and Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan

Online publication date: 19 September 2000

To cite this Article Lu, Kuo -Liang , Ku, Yoe -Ray , Wen, Kuo -Ching , Ho, Li -Kang and Chang, Yuan -Shiun(2000) 'ANALYSIS OF FLAVONOIDS AND COUMARINS IN *IXERIS LAEVIGATA* VAR. *OLDHAMI* BY HPLC', Journal of Liquid Chromatography & Related Technologies, 23: 16, 2573 — 2583
To link to this Article: DOI: 10.1081/JLC-100100511
URL: http://dx.doi.org/10.1081/JLC-100100511

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 23(16), 2573–2583 (2000)

ANALYSIS OF FLAVONOIDS AND COUMARINS IN *IXERIS LAEVIGATA* VAR. *OLDHAMI* BY HPLC

Kuo-Liang Lu,[1,3] Yoe-Ray Ku,[1,2] Kuo-Ching Wen,[2] Li-Kang Ho,[4] Yuan-Shiun Chang[1,*]

[1] Institute of Chinese Pharmaceutical Sciences
China Medical College
91, Hsueh-Shih Road
Taichung 404, Taiwan

[2] National Laboratories of Foods and Drugs
Department of Health
Executive Yuan
161-2, Kuen-Yang Street
Nankang, Taipei 115, Taiwan

[3] Chung Hwa Institute of Technology
89, Wen-Hwa 1st Street
Jen-Teh Hsiang
Tainan Hsien 717, Taiwan

[4] Department and Institute of Pharmacology
National Yang-Ming University
155, Sec. 2, Li-Nung Street
Shih-Pai, Pettou, Taipei 112, Taiwan

Downloaded At: 09:46 24 January 2011

ABSTRACT

Tao-shang-tsao is the dried entire plant of *Ixeris laevigata* var. *oldhami* (Compositae) and a commonly used folk herb in Taiwan. To evaluate the quality of *I. laevigata* var. *oldhami*, a simple, rapid, and accurate high-performance liquid chrom-atographic (HPLC) method was developed for the assay of four flavonoids,

Copyright © 2000 by Marcel Dekker, Inc. www.dekker.com

apigenin, apigenin-7-*O*-glucoside, luteolin, and luteolin-7-*O*-glucoside, and two coumarins, esculetin and esculin.

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. Ethylparaben 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 ethylparaben. The relative standard deviations of these six constituents ranged between 0.56-3.04% (intra-day) and 0.79-4.39% (inter-day). The contents of six constituents of *I. laevigata* var. *oldhami* in 7 crude drugs have been determined.

This study also compared their constituents with those of similar folk herbs, *I. chinensis* and *Taraxacum formosanum*, commonly used as an adulterant, by their HPLC chromatograms.

Downloaded At: 09:46 24 January 2011

INTRODUCTION

Traditional Chinese medicinal herbs have been used for over 1500 years. Most of them are composed of complex chemical constituents. Proper methods are required for quality control of traditional Chinese medicines, using effective chromatographic methods. In our laboratory, we have developed several high-performance liquid chromatographic (HPLC) methods for the determination of marker constituents in traditional Chinese medicines.[1-5]

Tao-shang-tsao is the dried entire plant of *Ixeris laevigata* var. *oldhami* (Compositae) and a commonly used folk herb in Taiwan. It possesses anti-inflammatory and antibacterial effects and is used to treat cold, cough, asthma, and hepatitis.[6-7] Four flavonoids: apigenin (API), apigenin-7-*O*-glucoside (APG), luteolin (LUT), and luteolin-7-*O*-glucoside (LUG), and two coumarins: esculetin (ESE) and esculin (ESC), have been isolated from the plant.[8] Flavonoids and coumarins have shown many biological and pharmacological activities,[9-11] and could be used as markers for chemical evaluation. The volume of sales of folk herbs tends to increase in Taiwan, therefore, the quality control for folk herbs is needed. *I. chinensis* and *Taraxacum formosanum* are two folk herbs similar to *I. laevigata* var. *oldhami*. These three herbs are commonly used as adulterants to one another.[12]

In general, the identification of medicinal herbs is usually based on the apparent characteristics and the examination of herbal tissue under light microscope. However, those procedures are difficult and tedious. HPLC is a more promising technique because it allows high resolution and a rapid and repro-

ducible determination, even of trace amounts of compounds. On the other hand, a simple and rapid method is needed to be applied to routine analysis of plants. In this study, we developed an HPLC method for the determination of the above six constituents of *I. laevigata* var. *oldhami.* The contents of six constituents in seven samples from markets have been determined. Additionally, comparisons were made among *I. laevigata* var. *oldhami, I. chinensis,* and *T. formosanum* by their HPLC chromatograms.

EXPERIMENTAL

Reagents and Materials

APG, API, ESC, ESE, LUG, and LUT were isolated from the entire plant of *I. laevigata* var. *oldhami*.[8] Ethylparaben 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. Seven, eight, and two samples of *I. laevigata* var. *oldhami*, *I. chinensis,* and *Taraxacum formosanum*, respectively, were obtained from markets in Taipei, Taichung and Tainan of Taiwan. All samples were identified by comparative anatomical studies.[12]

Downloaded At: 09:46 24 January 2011

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 solvent gradient of A-B [A=acetonitrile; B=0.1% (v/v) phosphoric acid] varying as follows: 0 min, 10:90; 12 min, 30:70; and 32 min, 40:60.

Preparation of Standard Solution

To prepare standard solutions (containing APG, API, ESC, ESE, LUG, and LUT), an appropriate amount of internal standard solution was added to an accurately weighed amount of APG, API, ESC, ESE, LUG, and LUT standard, which was dissolved in 70% methanol for HPLC. The various concentrations were within the range 2.6-41.6, 4.5-72.0, 5.0-80.0, 5.0-80.0, 2.7-43.2, and 3.6-57.6 μg/mL, respectively. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with concentrations.

Preparation of Sample Solution: Extraction Solvent Study

Twenty grams of *I. laevigata* var. *oldhami* was cut to pieces and mixed well. Two grams of this sample was extracted two times (15 and 12 mL, successively) with various ratios (50, 70, 85, and 100%) of methanol by reflux at 80°C, each one hour. The extracts were combined and filtered into a volumetric flask; 70% methanol was added to 25 mL and used as stock solution. The sample solution was prepared by 2.5 mL of above solution and 0.5 mL of ethylparaben solution (200 μg/mL) into a 5 mL volumetric flask and then adjusting the volume to 5 mL, exactly, with 70% methanol. This solution was filtered through a 0.45 μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA) before use.

Sample Determination

Two grams of each *I. laevigata* var. *oldhami* sample was extracted two times (15 and 12 mL, successively) with 70% methanol and then processed as above.

Downloaded At: 09:46 24 January 2011

Preparation of Recovery Studies

Three different concentrations of markers: 2.1, 4.2, and 8.3 μg/mL for APG; 3.6, 7.2, and 14.4 μg/mL for API; 4.0, 8.0, and 16.0 μg/mL for ESC; 4.0, 8.0, and 16.0 μg/mL for ESE; 2.2, 4.3, and 8.6 μg/mL for LUG; and 2.9, 5.8, and 11.5 μg/mL for LUT were added to each sample solution, respectively. To each solution, a suitable amount of internal standard was added to yield a final concentration of 20.0 μg/mL of ethylparaben. All samples were filtered through a 0.45 μm syringe filter (Gelman) and injected for HPLC analysis to calculate the concentration of APG, API, ESC, ESE, LUG, and LUT from their calibration graphs.

RESULTS AND DISCUSSION

The detection wavelength of 254 nm was chosen because these six constituents have better absorption at this wavelength. The photodiode array detection facilitated the identification and confirmation of these six constituents. Figure 1 presents a chromatogram showing the separation of the constituents with the retention times of 26.3 min for the internal standard (ethylparaben); 8.7 min for ESC; 12.0 min for ESE; 15.2 min for LUG; 17.0 min for APG; 22.9 min for LUT; and 27.7 min for API. When the sample solution was injected directly and analyzed, the whole analysis was finished 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, 5.0-80.0 μg/mL for ESC, 5.0-80.0 μg/mL for

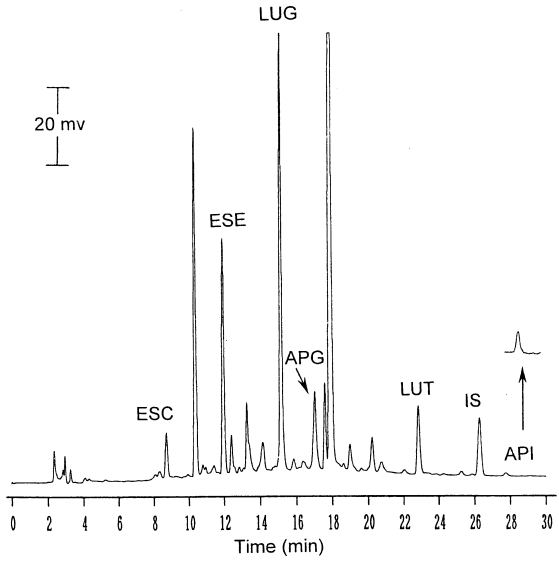


Figure 1. Chromatogram of 70 % methanol extract of *I. laevigata* var. *oldhami*. APG, apigenin-7-O-glucoside; API, apigenin; ESC, esculin; ESE, esculetin; LUG, luteolin-7-O-glucoside; LUT, luteolin; IS= internal standard (ethylparaben); HPLC conditions, column: Inertsil ODS-2, 5 μm, 25 cm × 4.6 mm I.D.; 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.

Downloaded At: 09:46 24 January 2011

ESE, 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=76.82x+1.02 (r=0.9999); API, y=35.51x+0.97 (r=0.9999); ESC, y=79.98x+0.96 (r=0.9998); ESE, y=36.68x+0.89 (r=0.9999); LUG, y=53.50x+1.07 (r=0.9999); and LUT, y=27.04x+1.27 (r=0.9999). It showed good linear relationships between the peak area ratios and the concentrations. A signal three times higher than the peak noise height was regarded as the detection limit. The detection limits of these six constituents were: 0.2, 0.6, 0.3, 0.2, 0.6, and 0.4 μg/mL for APG, API, ESC, ESE, LUG, and LUT, respectively.

To assess the precision of these methods, we injected standard solutions of APG, API, ESC, ESE, LUG, and LUT, respectively, six times on the same day and a 6-day period analysis. The coefficient variations of intra-day and inter-day studies were less than 3.5 and 4.5%, respectively. The precision, as well as, accuracy of this assay was satisfactory (Table 1). The results for the

Table 1

Intra-Day and Inter-Day Assay Variations of APG, API, ESC, ESE, LUG, and LUT

Constituent	Concentration (μg/mL)	Intra-Day* R.S.D. (%)	Inter-Day* R.S.D. (%)
	2.6	2.57	2.70
APG	10.4	0.89	1.53
	41.6	0.93	1.15
	4.5	2.78	1.78
API	18.0	0.58	1.32
	72.0	0.59	0.79
	5.0	2.49	3.90
ESC	20.0	2.05	1.72
	80.0	0.69	0.68
	5.0	1.22	4.39
ESE	20.0	2.56	1.11
	80.0	0.68	1.32
	2.7	1.75	1.94
LUG	10.8	0.95	1.09
	43.2	0.76	0.95
	3.6	3.04	3.05
LUT	14.4	1.45	0.79
	57.6	0.56	0.47

* n = 6.

Downloaded At: 09:46 24 January 2011

recoveries of APG, API, ESC, ESE, LUG, and LUT ranged from 95.1 to 102.5% (Table 2). The R.S.D.s of recoveries of six constituents ranged between 0.5-1.7%.

When the sample solution was analyzed by HPLC, the peaks were identified by comparison of the retention time with those obtained from authentic samples of *I. laevigata* var. *oldhami*. The extraction yields of six constituents using different ratios of methanol were shown in Figure 2. With 70% methanol, except for two coumarins, four flavonoids yielded the best extraction rates. Therefore, 70% methanol was used as extraction solvent throughout this study. The comparison graph of contents of the constituents mentioned above in 7 crude drugs of *I. laevigata* var. *oldhami* are given in Figure 3.

Table 2

Recoveries of APG, API, ESC, ESE, LUG, and LUT in *I. laevigata* var *oldhami*

Constituent	Amount Added (μg/mL)	Recovery (%)	Mean ± S.D. (%)	R.S.D. (%)
	2.1	97.6		
APG	4.2	98.5	98.8 ± 1.1	1.1
	8.3	100.3		
	3.6	99.8		
API	7.2	101.5	99.9 ± 1.3	1.3
	14.4	98.4		
	4.0	96.9		
ESC	8.0	97.2	97.8 ± 1.1	1.1
	16.0	99.3		
	4.0	95.3		
ESE	8.0	96.2	95.5 ± 0.5	0.5
	16.0	95.1		
	2.2	97.5		
LUG	4.3	98.3	98.1 ± 0.5	0.5
	8.6	98.6		
	2.9	98.3		
LUT	5.8	100.4	100.4 ± 1.7	1.7
	11.5	102.5		

* n = 3

Downloaded At: 09:46 24 January 2011

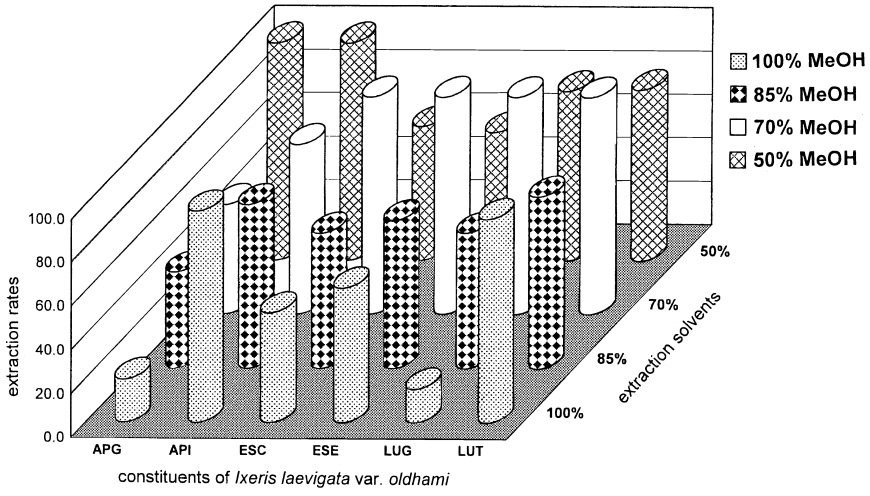


Figure 2. Comparison of extraction ratios of six constituents of *I. laevigata* var. *oldhami* by various solvents. For abbreviations, see Figure 1.

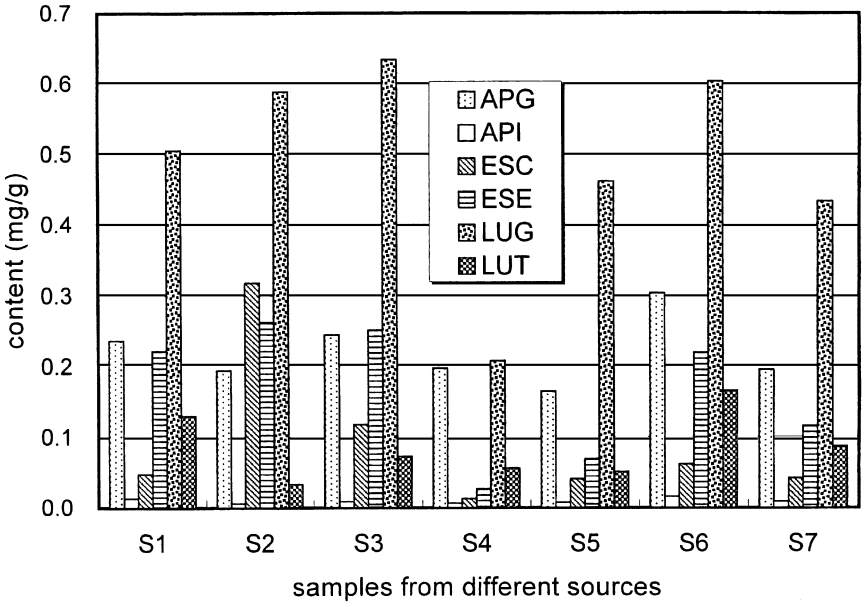


Figure 3. The contents of six constituents in seven *I. laevigata* var. *oldhami* (S1-S7) obtained from different sources. For abbreviations, see Figure 1.

Downloaded At: 09:46 24 January 2011

The contents of six constituents ranged between 0.1646-0.3033, 0.0058-0.0154, 0.0124-0.3176, 0.0262-0.2619, 0.2076-0.6339, and 0.0506-0.1655 mg/g for APG, API, ESC, ESE, LUG, and LUT, respectively, were extracted from crude drugs. The extraction rates of APG and LUG (flavonoid glycoside) were higher than API and LUT (aglycone). It revealed that major flavonoids consisted of the glycoside type. The total coumarins and total flavonoids ranged between 0.0386-0.5795 mg/g and 0.4663-1.0872 mg/g, respectively.

References[13-15] and Figure 4 showed that APG, LUG, and LUT were contained in *I. chinensis* and *T. formosanum*. We could easily differentiate among

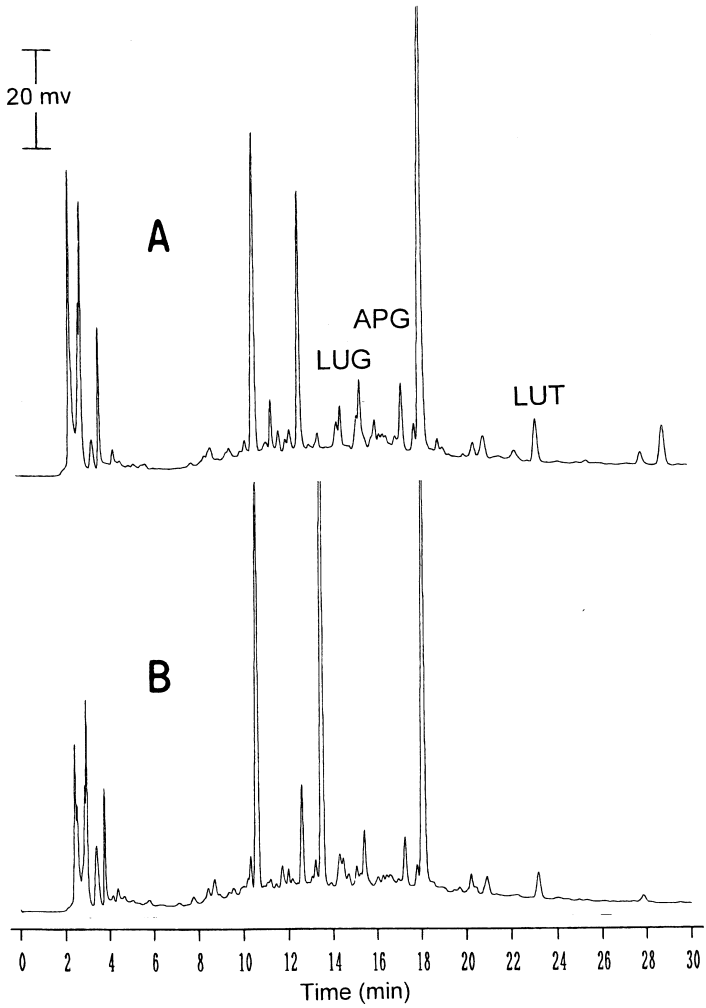


Figure 4. Chromatograms of *I. chinensis* (A) and *T. formosanum* (B). For HPLC conditions and abbreviations, see Figure 1.

the chromatograms of *I. laevigata* var. *oldhami*, *I. chinensis* and *T. formosanum*. Since there are differences in contents of constituents among these three herbs, it is suggested that identification steps should be taken before medical uses.

Downloaded At: 09:46 24 January 2011

REFERENCES

1. S.-J. Lin, H.-H. Tseng, K.-C. Wen, T.-T. Suen, J. Chromatogr. A, **730**, 17 (1996).

2. Y.-R. Ku, Y.-T. Lin, K.-C. Wen, J.-H. Lin, C.-H. Liao, J. Liq. Chrom. & Rel. Technol., **19**, 3265 (1996).

3. J.-H. Lin, Y.-R. Ku, Y.-S. Huang, K.-C. Wen, C.-H. Liao, J. Liq. Chrom. & Rel. Technol., **20**, 1617 (1997).

4. K.-C. Wen, H.-H. Tseng, L. Chin, K.-S. Huang, F.-L. Lu, F.-S. Liu, S.-J. Lin, Y.-R. Ku, Y.-T. Lin, **Determination of Marker Constituents in Traditional Chinese Medicinal Prescriptions**, National Laboratories of Foods and Drugs, Taipei, 1999, pp. 1-154.

5. Y.-R. Ku, K.-C. Wen, L.-K. Ho, Y.-S. Chang, J. Pharm. Biomed. Anal., **20**, 351 (1999).

6. H.-C. Chang, **Herbal Folk Medicines**, Green Life Publications, Inc., Taipei, 1996, pp. 62-63.

7. K.-L. Lu, C.-C. Tsai, L.-K. Ho, Y.-S. Chang, The 14th Symposium on Natural Product, 1999, p. 218.

8. L.-K. Ho, K.-L. Lu, S.-C. Huang, Chemistry (The Chinese Chem. Soc., Taiwan China), **47**, 134 (1989).

9. B. Havesteen, Biochem. Pharmacol., **32**, 1141 (1983).

10. W. Bors, W. Heller, C. Michel, M. Saran, Methods Enzymol., **186**, 343 (1990).

11. H. Nishino, M. Nagao, H. Fujiki, T. Sugimura, Cancer Lett., **21**, 1 (1983).

12. Y.-H. Hsu, L. Lai, H.-H. Tseng, H.-C. Chang, K.-C. Wen, Ann. Rept. NLFD, **16**, 51 (1998).

13. Y.-L. Huang, M.S.D. Thesis, Chinese Culture University, Taipei, Taiwan (1986).

14. S.-J. Cheng, M.S.D. Thesis, China Medical College, Taichung, Taiwan (1996).

Downloaded At: 09:46 24 January 2011

15. H.-L. Chou, J.-R. Yaun, Zhongcaoyao (Chinese Traditional Herbal Drug), **27**, 267 (1996).

Received February 10, 2000 Author's Revisions May 29, 2000
Accepted February 25, 2000 Manuscript 5249

Downloaded At: 09:46 24 January 2011