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Analysis of three lupane type triterpenoids in *Helicteres angustifolia* by high-performance liquid chromatography

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Abstract

Kang-chih-ma is the dried roots and stems of *Helicteres angustifolia* (Sterculiaceae) and a commonly used folk herbal drug in Taiwan. It possesses anti-dotal, analgesic, anti-inflammatory and anti-bacterial effects and is also known as a kind of tumor inhibitory plant. To evaluate the quality of *H. angustifolia*, a simple, rapid and accurate high-performance liquid chromatographic (HPLC) method was developed for the assay of three lupane type triterpenes: 3β-acetoxy-27-benzoyloxylup-20(29)-en-28-oic acid methyl ester (methyl helicterate), 3β-acetoxy-27-benzoyloxylup-20(29)-en-28-oic acid, and 3β-acetoxy-27-(*p*-hydroxyl) benzoyloxylup-20(29)-en-28-oic acid methyl ester. The present HPLC system used an Inertsil ODS-2 column by gradient elution with acetonitrile and water as the mobile phase and detected at UV 230 nm. Regression equations revealed good linear relationships (correlation coefficients: 0.9922–0.9997). The relative standard deviations of these three constituents ranged between 1.05–3.14% (intraday) and 2.12–4.38% (interday). The contents of these three constituents of the heartwood and the bark of the roots of *H. angustifolia* in five different samples have also been determined.

Keywords: Pharmaceutical analysis; *Helicteres angustifolia*; Methyl helicterate

1. Introduction

Traditional Chinese medicinal herbs have been used for centuries. Most of them are composed of complex chemical constituents. Proper methods are required for quality control of traditional Chinese medicines, using effective chromato-

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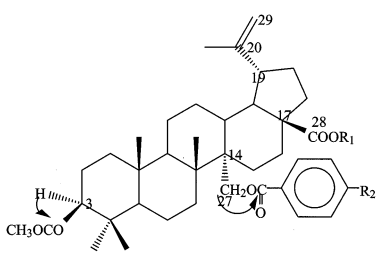
graphic methods. In our laboratory, we have developed several high-performance liquid chromatographic (HPLC) [1–4] and capillary electrophoresis [5,6] methods for the determination of marker constituents in traditional Chinese medicines and folk herbs.

Kang-chih-ma is the dried roots of *Helicteres angustifolia* (Sterculiaceae) and a commonly used folk herbal drug in Taiwan. It possesses antidotal, analgesic, anti-inflammatory and anti-bacterial effects and is also known as a kind of tumor inhibitory plant [7]. Several sesquiterpenes and triterpenes have been isolated from this plant [8–10]. In the pharmacological test, the *n*-hexane layer, CH_2Cl_2 layer, EtOAc layer and *n*-BuOH layer of methanol extract from the roots of this plant were found to have potent cytotoxic activity [11]. This plant is one of the substitutions or adulterations of Shan-Dou-Gen (*Sophora subprosprata*, Leguminosae) on the market. The latter medicinal herb also possesses anti-inflammatory and anti-bacterial actions and is indicated in sore throat, acute pharyngolaryngeal infections and gingivitis [12]. Three lupane type triterpenes: 3β-acetoxy-27-benzoyloxylup-20(29)-en-28-oic acid methyl ester (methyl helicterate; HA-1) [8–10], 3β-acetoxy-27-benzoyloxylup-20(29)-en-28-oic acid (HA-2) [9,10], and 3β-acetoxy-27-(*p*-hydroxyl) benzoyloxylup-20(29)-en-28-oic acid methyl ester (HA-3) [10], have been isolated from the plant by our laboratory and chemical structures were identified by spectroscopic methods (Fig. 1). Most triterpenes are important because of their biological activity [13], and could be used as markers for chemical evaluation. The volume of sales of folk herbal drugs tends to increase in Taiwan, therefore, the quality control for folk herbal drugs is needed.



HA-1 R_1= CH_3 R_2= H
HA-2 R_1= H R_2= H
HA-3 R_1= CH_3 R_2= OH

Fig. 1. The chemical structures of three marker constituents.

The troublesome process of separating a pure compound from crude extracts obtained from plant results in difficulty determining the quantities and characterizing active ingredients. Because of the sensitivity at used wavelength and high detection limit due to the background interference of terpenoids. However, the benzoic acid methyl ester group in these three triterpenes provided an UV absorption at 230 nm, which solved the above problem.

In this study, we developed an HPLC method for the determination of these triterpenes of *H. angustifolia*. The contents of these three constituents in five samples from Taiwan markets had been determined.

2. Experimental

2.1. Plant material

Plant materials of *H. angustifolia* were collected in Taichung Hsein in September, 1997 and identified by Mr Nien-Yung Chiu, Institute of Chinese Pharmaceutical Science, China Medical College, Taichung, Taiwan. A voucher specimen (No. 970903) was deposited in the herbarium of the Institute of Chinese Pharmaceutical Science, China Medical College. Five samples of *H. angustifolia* were obtained from markets in Taipei and Taichung of Taiwan. All samples were identified by comparative microscopy studies.

2.2. Reagents

HA-1, -2 and -3 were isolated from the dried roots of *H. angustifolia*. Acetonitrile (HPLC grade) was purchased from Labscan (Dublin, Ireland). Ultra-pure distilled water with a resistance greater than 18 MΩ was used. Methanol, chloroform, dichloromethane, ethylacetate, *n*-butanol and *n*-hexane were industrial grade or analytical grade.

2.3. Apparatus

Melting points were measured on a Yanaco MP-500 and were uncorrected. Mass spectra were obtained using a VG Platform II mass spectrometer with a direct probe (70 eV). ^{1}H and ^{13}C NMR spectra were recorded on Bruker DMX-500SB and Varian VXR-300 FT-spectrometers (National Chung Hsing University, Taichung).

2.4. Extraction and isolation

The air-dried stems and roots of *H. angustifolia* (10 kg) were extracted exhaustively with MeOH at room temperature (30 l × 3) 7 days each time. Then, the extract was concentrated under reduced pressure. The crude MeOH extract (He-M, 650 g) was suspended in water and partitioned successively with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH subsequently, to afford the hexane fraction (He-H, 45 g), CH_2Cl_2 fraction (He-C, 105 g), EtOAc fraction (He-E, 14 g), *n*-BuOH fraction (He-B, 195 g) and water fraction (He-W, 284 g). He-C was chromatographed on silica gel eluting with *n*-hexane–acetone mixtures of increasing polarity to yield HA-1, -2 and -3, respectively.

HA-1 [3β-acetoxy-27-benzoyloxylup-20(29)-en-28-oic acid methyl ester (methyl helicterate)]:colorless needles (CH_2Cl_2–MeOH), m.p. 196–197 °C, mass (70 eV) *m/z*: 632 (M^+, $C_{40}H_{56}O_6$), ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.98 (2H, dd, $J = 7.5$, 2.5 Hz, H-2′,6′), 7.54 (1H, m, H-4′), 7.43 (2H, m, H-3′, 5′), 4.75 (1H, d, $J = 12.8$ Hz, H-27a), 4.73 (1H, d, $J = 1.6$ Hz, H-29a), 4.60 (1H, d, $J = 1.6$ Hz, H-29b), 4.53 (1H, d, $J = 12.8$ Hz, H-27b), 4.42 (1H, dd, $J = 11.4$, 5.0 Hz, H-3α), 3.66 (3H, s, 17-$COOCH_3$), 3.00 (1H, m, H-19), 1.99 (3H, s, 3-$OCOCH_3$), 1.68 (3H, s, H-30), 0.99 (3H, s, H-24), 0.87 (3H, s, H-25), 0.79 (3H, s, H-26), 0.77 (3H, s, H-23), ^{13}C NMR ($CDCl_3$, 125 MHz): δ 38.46 (t, C-1), 23.63 (t, C-2), 80.64 (d, C-3), 37.78 (s, C-4), 55.50 (d, C-5), 18.12 (t, C-6), 35.27 (t, C-7), 41.48 (s, C-8), 51.96 (d, C-9), 37.39 (s, C-10), 21.05 (t, C-11), 25.24 (t, C-12), 39.03 (d, C-13), 45.66 (s, C-14), 24.22 (t, C-15), 32.48 (t, C-16), 56.29 (s, C-17), 49.76 (d, C-18), 46.83 (d, C-19), 150.07 (s, C-20), 30.38 (t, C-21), 36.53 (t, C-22), 27.83 (q, C-23), 16.68 (q, C-24), 16.53 (q, C-25), 16.34 (q, C-26), 63.64 (t, C-27), 176.57 (s, C-28), 109.96 (t, C-29), 19.41 (q, C-30), 170.80 (s, 3-$OCOCH_3$), 23.63 (q, 3-$OCOCH_3$), 51.33 (q, 28-$COOCH_3$), 166.52 (s, 27-CH_2OCO–), 130.57 (s, C-1′), 129.42 (d, C-2′), 128.45 (d, C-3′), 132.89 (d, C-4′), 128.45 (d, C-5′), 129.42 (d, C-6′). These assignments were based on the result from a combination of DEPT, HMQC and HMBC. The HMQC experiment was used to determine the ^{1}H and ^{13}C NMR vicinal correlation, and the HMBC technique confirmed the characteristic long-change correlation that C-3 acetyl group, C-28 methyl ester and C-27 attached the benzoyloxy ester group.

HA-2 [3β-acetoxy-27-benzoyloxylup-20(29)-en-28-oic acid]: colorless needles (CH_2Cl_2–MeOH), m.p. > 300 °C, mass (70 eV) *m/z*: 618 (M^+, $C_{39}H_{54}O_6$), ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.00 (2H, d, $J = 7.5$ Hz, H-2′,6′), 7.49 (3H, m, H-3′,4′,5′), 4.75 and 4.62 (each 1H, s, H-29), 4.78–4.53 (2H, m, H-27), 4.44 (1H, dd, $J = 10.7$, 5.0 Hz, H-3α), 3.03 (1H, m, H-19), 2.40–2.30 (2H, m), 2.00 (3H, s, 3-$OCOCH_3$), 1.70 (3H, s, H-30), 1.01 (3H, s, H-24), 0.89 (3H, s, H-25), 0.81 (3H, s, H-26), 0.78 (3H, s, H-23), ^{13}C NMR ($CDCl_3$, 75 MHz): δ 38.42 (t, C-1), 23.65 (t, C-2), 80.68 (d, C-3), 37.80 (s, C-4), 55.50 (d, C-5), 18.14 (t, C-6), 35.26 (t, C-7), 41.53 (s, C-8), 51.89 (d, C-9), 37.43 (s, C-10), 21.04 (t, C-11), 25.24 (t, C-12), 39.24 (d, C-13), 45.73 (s, C-14), 24.29 (t, C-15), 32.53 (t, C-16), 56.20 (s, C-17), 49.57 (d, C-18), 46.85 (d, C-19), 149.90 (s, C-20), 30.41 (t, C-21), 32.70 (t, C-22), 27.86 (q, C-23), 16.69 (q, C-24), 16.54 (q, C-25), 16.44 (q, C-26), 63.67 (t, C-27), 182.16 (s, C-28), 110.09 (t, C-29), 19.43 (q, C-30), 170.88 (s, 3-$OCOCH_3$), 21.22 (q, 3-$OCOCH_3$), 166.56 (s, 27-CH_2OCO–), 130.55 (s, C-1′), 129.45 (d, C-2′), 128.48 (d, C-3′), 132.94 (d, C-4′), 128.48 (d, C-5′), 129.45 (d, C-6′). In comparison with HA-1, the signal of δ_H 3.66 (17-$COOCH_3$) and δ_C 51.34 (17-$COOCH_3$) in HA-2 were absent, the structure was then elucidated.

HA-3 [3β-acetoxy-27-(*p*-hydroxyl)-benzoyloxy-lup-20(29)-en-28-oic acid methyl ester]: colorless needles (CH_2Cl_2–MeOH), m.p. > 300 °C, mass (70 eV) *m/z*: 648 (M^+, $C_{40}H_{56}O_7$), ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.85 (2H, d, $J = 8.0$ Hz, H-2′,6′), 6.85 (2H, d, $J = 8.0$ Hz, H-3′,5′), 3.66

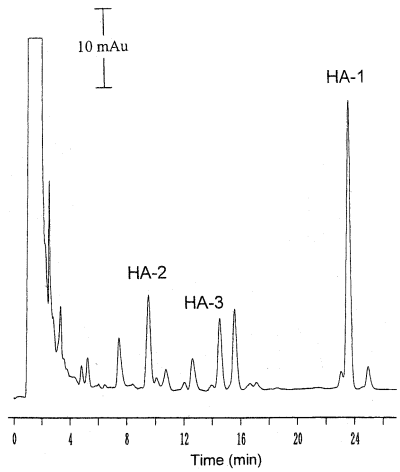


Fig. 2. Chromatogram of methanol extract of *H. angustifolia*. HA-1: 3β-acetoxy-27-benzoyloxylup-20(29)-en-28-oic acid methyl ester, HA-2: 3β-acetoxy-27-benzoyloxylup-20(29)-en-28-oic acid, HA-3: 3β-acetoxy-27-(*p*-hydroxyl)-benzoyloxylup-20(29)-en-28-oic acid methyl ester. HPLC conditions, column: Inertsil ODS-2, 5 μm, 25 cm × 4.6 mm i.d.; mobile phase: A–B (A, acetonitrile; B, water), 0 min, 85:15 and 25 min, 100:0; flow rate: 1.5 ml/min; detection wavelength: 230 nm.

(3H, s, 17-$COOCH_3$), 3.00 (1H, m, H-19), 2.01 (3H, s, 3-$OCOCH_3$), 1.67 (3H, s, H-30), 1.00 (3H, s, H-24), 0.86 (3H, s, H-25), 0.80 (3H, s, H-26), 0.77 (3H, s, H-23), ^{13}C NMR ($CDCl_3$, 75 MHz): δ 38.49 (t, C-1), 23.68 (t, C-2), 80.93 (d, C-3), 37.84 (s, C-4), 55.52 (d, C-5), 18.19 (t, C-6), 35.30 (t, C-7), 41.53 (s, C-8), 51.95 (d, C-9), 37.45 (s, C-10), 21.11 (t, C-11), 25.32 (t, C-12), 39.11 (d, C-13), 45.73 (s, C-14), 24.39 (t, C-15), 32.50 (t, C-16), 56.40 (s, C-17), 49.77 (d, C-18), 46.92 (d, C-19), 150.13 (s, C-20), 30.46 (t, C-21), 32.60 (t, C-22), 27.90 (q, C-23), 16.73 (q, C-24), 16.56 (q, C-25), 16.41 (q, C-26), 63.43 (t, C-27), 176.77 (s, C-28), 109.97 (t, C-29), 19.45 (q, C-30), 171.59 (s, 3-$OCOCH_3$), 21.28 (q, 3-$OCOCH_3$), 51.54 (q, 28-$COOCH_3$), 166.44 (s, 27-CH_2OCO–), 122.53 (s, C-1′), 131.75 (d, C-2′), 115.36 (d, C-3′), 160.32 (d, C-4′), 115.36 (d, C-5′), 131.75 (d, C-6′). In comparison with HA-1, the structure showed no difference except the presence of A_2X_2 type *p*-hydroxyl substituted.

2.5. High-performance liquid chromatography system

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 230 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 1.5 ml/min with a linear solvent gradient of A–B (A, acetonitrile; B, water) varying as follows: 0 min, 85:15 and 25 min, 100:0.

2.6. Preparation of standard solution

To prepare standard solutions (containing HA-1, -2 and -3), an accurately weighed amount of

Table 1
Intraday and interday assay variations of three marker constituents

Constituent	Concentration (μg/ml)	Intraday[a] R.S.D. (%)	Interday[a] R.S.D. (%)
HA-1	5.0	2.98	3.78
	20.0	2.01	4.03
	80.0	1.05	3.16
HA-2	7.2	2.05	4.11
	28.9	3.14	4.38
	86.6	2.70	2.89
HA-3	5.2	2.16	3.72
	20.6	3.01	3.07
	41.2	2.65	2.12

[a] $n = 6$.

Table 2
Recoveries of three marker constituents in *H. angustifolia*

Constituent	Amount added (μg/ml)	Recovery* (%)	Mean ± SD (%)	R.S.D. (%)
HA-1	5.0	96.8	95.1 ± 2.7	2.8
	10.0	97.2		
	20.0	91.3		
HA-2	7.2	94.5	92.5 ± 2.4	2.5
	14.4	93.8		
	28.9	89.2		
HA-3	5.2	87.2	91.9 ± 4.3	4.6
	10.3	91.0		
	20.6	97.5		

*$n = 3$.

HA-1, -2 and -3 standard which was dissolved in methanol for HPLC. Five concentrations were chosen, with the range 5.0–80.0, 7.2–86.6 and 5.2–41.2 μg/ml respectively. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with concentrations.

2.7. Preparation of sample solution

2.7.1. Extraction solvent study

Twenty grams of the roots of *H. angustifolia* were cut to pieces and mixed well. Four grams of this sample was extracted two times (25 and 20 ml, successively) with methanol, EtOAc, *n*-hexane, chloroform and dichloromethane by reflux at 80 °C, each 1 h. The extracts were combined and filtered while hot. The filtrate was concentrated under reduced pressure to dryness. The residue was dissolved in 10 ml of methanol. This solution was filtered through a 0.45-μm syringe filter (Gelman Sciences, Ann Arbor, MI) before use.

2.7.2. Sample determination

Four grams of the bark and wood of the root of each *H. angustifolia* sample was extracted two times (25 and 20 ml, successively) with methanol and then processed as above.

2.8. Preparation of recovery studies

Three different concentrations of markers: 5.0, 10.0 and 20.0 μg/ml for HA-1, 7.2, 14.4 and 28.9 μg/ml for HA-2 and 5.2, 10.3 and 20.6 μg/ml for HA-3 were added to the plant material samples, respectively. All samples were filtered through a 0.45-μm syringe filter (Gelman) and injected for HPLC analysis to calculate the concentration of HA-1, -2 and -3 from their calibration graphs.

3. Results and discussion

The detection wavelength of 230 nm was chosen because these three constituents have better absorption at this wavelength. The photodiode array detection facilitated the identification and confirmation of these three constituents. Fig. 2

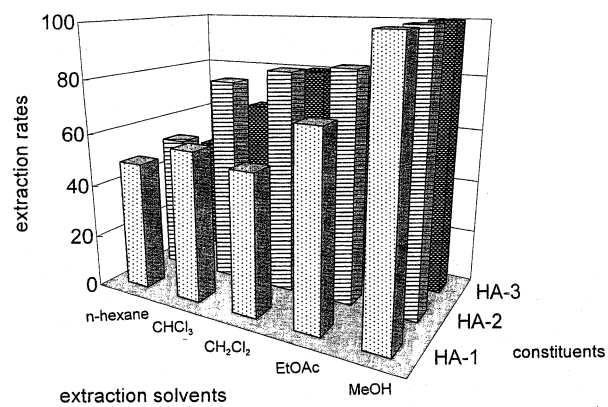


Fig. 3. Comparison of extraction solvents of three constituents of *H. angustifolia* by various solvents. For abbreviations, see Fig. 2.

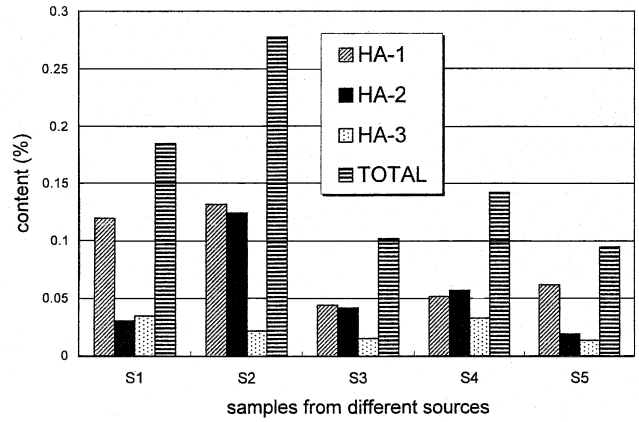


Fig. 4. The contents of three constituents in five *H. angustifolia* (S1–S6) obtained from different sources. For abbreviations, see Fig. 2.

presented a chromatogram showing the separation of the constituents with the retention times of 9.5 min for HA-2; 14.5 min for HA-3 and 23.5 min for HA-1. When the sample solution was injected directly and analyzed, the whole analysis was finished within 25 min.

Calibration graphs were constructed in the range 5.0–80.0 μg/ml for HA-1, 7.2–86.6 μg/ml for HA-2 and 5.2–41.2 μg/ml for HA-3. The regression equations of these curves and their correlation coefficients (r), coefficients of determination (R^2) were calculated as follows: HA-1, $y = 3.96\text{E} - 04x - 6.64\text{E} - 02$, 0.9996, 99.83%; HA-2, $y = 3.29\text{E} - 04x - 0.18$, 0.9997, 99.82%, and HA-3, $y = 4.98\text{E} - 04x + 0.64$, 0.9922, 98.37%. It showed good linear relationships between the peak areas and the concentrations. A signal three times higher than the peak noise height was regarded as the detection limit. The detection limits of these three constituents were: 0.4, 0.7 and 1.3 μg/ml for HA-1, -2 and -3, respectively.

To assess the precision of these methods, we injected standard solutions of HA-1, -2 and -3, respectively, six times on the same day and a 6-day period analysis. The coefficient variations of intraday and interday studies were less than 4.0 and 5.0%, respectively. The precision as well as accuracy of this assay was satisfactory (Table 1). The results for the recoveries of HA-1, -2 and -3 ranged from 91.9 to 95.1% (Table 2). The relative standard deviations (R.S.D.s) of recoveries of three constituents ranged between 2.5 and 4.6%.

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 *H. angustifolia*. Since after two times extraction, the three constituents yielded over 98%. Therefore, two times extraction was chosen. The extraction yields of three constituents using different solvents were shown in Fig. 3. The extraction yields of different solvents were relative to the solvent polarities. The higher of the polarity of the solvent, the higher the yield was. With methanol, these three constituents yielded the best. Therefore, methanol was chosen for extraction throughout this study.

The heartwood and the bark of the roots were also separated for analysis. The results showed almost no detection of these three constituents in the heartwood of five different samples. The comparison was depicted of the contents of these five different samples (bark part) in Fig. 4. The contents of these three constituents ranged between 0.0444–0.1317%, 0.0191–0.1242% and 0.0135–0.0349% for HA-1, -2 and -3, respectively, were extracted from crude drugs. The total contents of HA-1, -2 and -3 ranged between 0.0904 and 0.2778%. It was found that the total content of these three triterpenes of S2 was 2–3 times higher than other samples.

In conclusion, we suggested that these three triterpenes is unique of Kang-chih-ma (*H. angustifolia*) and could be used as markers to determine the quality of *H. angustifolia*.

Acknowledgements

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