Quantitative Determination of Bioactive 4-Hydroxy-α-Tetralone, Tetralone-4-O-β-D-Glucopyranoside and Ellagic Acid in *Ammannia baccifera* (Linn.) by Reversed-Phase High-Performance Liquid Chromatography

Harish C. Upadhyay[1], Ram K. Verma[2] and Santosh K. Srivastava[1*]

[1]Medicinal Chemistry Department, and [2]Analytical Chemistry Department, Central Institute of Medicinal and Aromatic Plants, P.O.CIMAP, Lucknow−226015, India

*Author to whom correspondence should be addressed. Email: skscimap@gmail.com

Ammannia baccifera is an important component of various Chinese herbal formulations for which a rapid, simple, sensitive, gradient and reproducible reversed-phase high-performance liquid chromatographic method has been developed for the quantitative estimation of its bioactive constituents, 4-hydroxy-α-tetralone (**4H**), tetralone-4-O-β-D-glucopyranoside (**T4**) and ellagic acid (**EA**). The chromatographic separation of samples was performed on a Chromatopak Peerless C18 (250 × 4.6 mm i.d., 5 μm) column by gradient elution with 0.1% trifluoroacetic acid in water and methanol at a flow rate of 0.6 mL/min, a column temperature at 25°C and ultraviolet detection at λ 254 nm. The limit of detection (LOD) and limit of quantification (LOQ) were 1.51 and 5.06 μg/mL for EA, 0.70 and 2.33 μg/mL for T4 and 0.22 and 0.73 μg/mL for 4H, respectively. Good results were achieved with respect to linearity ($r^2 > 0.999$), repeatability (relative standard deviation $\leq 1.73\%$) and recovery (99.06−100.76%). The method was validated for linearity, accuracy, repeatability, LOQ and LOD. The method is simple, accurate and precise and was successfully applied to the analysis of these three analytes in five different leaf and root samples of *A. baccifera*; the method may be recommended for routine quality control analysis of various Chinese herbal formulations containing *A. baccifera*.

Introduction

Ammannia baccifera (Linn.) is widely used in traditional Chinese herbal formulations for treating human female infertility (1), gastroenteropathy (2), spinal disease (3), hemorrhoids (4), urethritis (5), common cold (6), abscess, sore, itching and other skin diseases (7, 8). It has been reported to possess anticancer, antirheumatic, antidiuretic, antipyretic, antisteroidogenic, antimicrobial and rubefacient activities (9). Recently, the ethanolic extract of *A. baccifera* has shown antiurolithic activity in male albino rats, by reducing formation of stones, primarily of magnesium ammonium phosphate with traces of calcium oxalate, and also dissolving pre-formed stones (10). *A. baccifera* belongs to the family Lythraceae and is commonly called "blistering Ammannia," "monarch red stem," "acrid weed" and "Bhatjambol." In Hindi, it is called "agni-buti," "ban mirich" and "jaungali mehndi." In India, it is commonly found in marshy places and paddy fields. The plants are erect and branched, and are annual herbs, growing up to 60 cm in height. Few reports have described the isolation of 4-hydroxy-α-tetralone (**4H**), ellagic acid (**EA**), betulinic acid and lupeol from *A. baccifera* (11, 12). **EA** widely occurs in its free form, as a glycoside or as

ellagitannins in the fruits, nuts and berries of several plants. **EA** possesses potential health-promoting benefits and antioxidant, antimutagenic, anti-inflammatory, hepatoprotective, cardioprotective and antiplasmodial activities (13, 14). Additionally, **4H** has shown potent anti-tuberculosis (15), anti-diabetic (16) and anti-leishmanial (17) activities. Recently, we isolated and characterized **4H** and its glucoside [tetralone-4-O-β-D-glucopyranoside (**T4**)] from *A. multiflora* and reported the bioenhancing activity of **4H** and its various semi-synthetic acyl derivatives (9).

In view of the potential bioactive properties of these isolated compounds (**4H**, **T4** and **EA**), they were used as a standard to develop a validated analytical procedure for the quality assurance of *A. baccifera*. To the best of our knowledge, there is no validated high-performance liquid chromatography (HPLC) method for the quantitative analysis of **4H** and its glucoside (**T4**) and **EA** in *A. baccifera*; however, many efforts have been made to separate and quantify ellagic acid in various other plants (18, 19). Hence, the objective of the present study was to optimize and develop a validated, rapid, sensitive and accurate reverse-phase HPLC method for the simultaneous determination and quantification of these bioactive molecules in the drug and herbal formulations containing *A. baccifera*. The present work is part of a series of efforts towards developing analytical methods (20, 21) for plant drug analysis.

Experimental

Plant material

The samples of *A. baccifera* were collected from five different districts of Uttar Pradesh, India in the month of October, 2010 and identified by Dr. D.C. Saini, Scientist, Birbal Shahni Institute of Palaeobotany (BSIP), Lucknow and Dr. S.C. Singh, Taxonomy and Pharmacognosy Division, Central Institute of Medicinal & Aromatic Plants (CIMAP), Lucknow, India. A voucher specimen, No. 9460, was deposited in the herbarium of CIMAP.

Isolation and characterization of reference compounds

The dried and pulverized roots (100 g) and aerial parts (600 g) of *A. baccifera* were separately extracted thrice with methanol (24 h each) at room temperature. The solvent was removed under reduced pressure, which afforded 13 and 80 g of methanolic extracts from the roots and aerial parts of *A. baccifera*, respectively. Because the thin-layer chromatography (TLC)
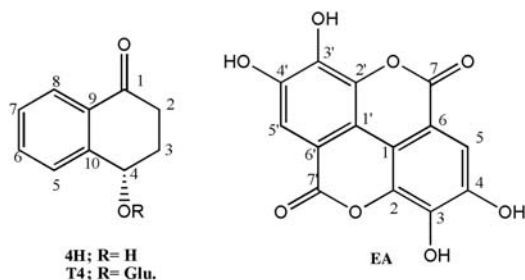
Figure 1. Structures of the isolated compounds: **4H**, **T4** and **EA**.

profile of both the extracts was more or less the same, they were pooled together. The pooled methanolic extract (93 g) was then dissolved in distilled water and successively partitioned with hexane, chloroform, and n-BuOH. The combined fractions were separately distilled under vacuum, which afforded hexane (28.0 g), chloroform (1.2 g) and n-BuOH (8.3 g) fractions.

Because the chloroform extract contained a major compound, a portion (1.0 g) of it was subjected to column chromatography (silica gel 60–120 mesh, 18 g, column diameter 1 × 31 cm). Gradient elution of the column was carried out with solvents of increasing polarity using hexane, chloroform and methanol. A total of 104 fractions of 50 mL each were collected and pooled on the basis of their TLC profile. Fractions 70–92 eluted with hexane–chloroform (10:90 to 2:98) afforded **4H** (380 mg). The butanol extract was a complex mixture; hence, 7 g of the extract was subjected to vacuum liquid chromatographic (VLC; 7.5 × 7.5 cm) separation over silica gel H (100 g), and stepwise gradient elution with chloroform and methanol was performed. A total of 325 fractions, each of 50 mL, were collected and pooled on the basis of their TLC profile. Fractions 114–123 eluted with chloroform–methanol (93:7) afforded **T4** (13.0 mg), while fractions 315–316 eluted with methanol afforded **EA** (8.2 g). Structures of isolated Compounds **4H**, **T4** and **EA** (Figure 1) were deduced on the basis of their 1D and 2D nuclear magnetic resonance (NMR) spectroscopic data, and the values were in agreement with those reported in literature (14, 16, 17).

4H (4-hydroxy-α-tetralone) yellowish amorphous solid. ^{1}H NMR (300 MHz, chloroform-d) δH: 2.44 (1H, m, H-2), 2.82 (1H, m, H-2), 2.36 (1H, m, H-3), 2.23 (1H, m, H-3), 4.67 (1H, d, $J =$ 6.2 Hz, H-4), 7.86 (1H, d, $J =$ 7.5 Hz, H-5), 7.47 (1H, dd, $J =$ 7.5, 1.5 Hz, H-6), 7.38 (1H, m, H-7), 7.27 (1H, dd, $J =$ 7.5, 1.5 Hz, H-8). ^{13}C NMR (75 MHz, chloroform-d) δC: 197.8 (C-1), 35.1 (C-2), 32.0 (C-3), 67.6 (C-4), 127.0 (C-5), 134.0 (C-6), 127.4 (C-7), 127.6 (C-8), 131.0 (C-9), 145.5 (C-10).

T4 (tetralone-4-O-β-D-glucopyranoside) amorphous powder. ^{1}H NMR (300 MHz, pyridine-d5) δH: 2.5 (1H, m, H-2), 2.9 (1H, m, H-2), 2.28 (1H, m, H-3), 2.39 (1H, m, H-3), 5.20 (1H, d, $J =$ 6.2 Hz, H-4), 7.91 (1H, d, $J =$ 7.5 Hz, H-5), 7.70 (1H, dd, $J =$ 7.5, 1.5 Hz, H-6), 7.42 (1H, m, H-7), 8.05 (1H, dd, $J =$ 7.5, 1.5 Hz, H-8); glucose; δ 4.51 (1H, d, $J =$ 7.5 Hz, H-1'), 3.33 (1H, m, H-2'), 3.32 (1H, m, H-3' and H-5'), 4.36 (1H, dd, $J =$ 11.7, 2.1 Hz, H-6'), 4.21 (1H, dd, $J =$ 11.7, 6.2 Hz, H-6'). ^{13}C NMR (75 MHz, pyridine-d5) δC: 197.2 (C-1), 35.0 (C-2), 31.9 (C-3), 75.1 (C-4) 127.0 (C-5), 133.4 (C-6), 129.1 (C-7), 128.6 (C-8),

132.0 (C-9), 143.4 (C-10); glucose; δ 104.0 (C-1'), 74.5 (C-2'), 78.4 (C-3'), 71.5 (C-4'), 78.3 (C-5'), 62.7 (C-6').

EA (Ellagic acid) colorless needles. ^{1}H NMR (300 MHz, DMSO-d6) δH: 7.40 (2H, s, H-5, 5'); ^{13}C NMR (75 MHz, DMSO-d6) δC: 107.7 (C-1,1'), 139.4 (C-2, 2'), 136.2 (C-3, 3'), 148.0 (C-4, 4'), 109.8 (C-5, 5'), 112.6 (C-6, 6'), 158.8 (C-7, 7').

Sample preparation

The dried and powdered leaf and root samples (1 g) were separately extracted with methanol–water (8:2) on an ultrasonic bath (3 × 25 mL, 30 min extraction time). The extracts were separately combined and solvent was removed under vacuum to dryness at 50°C. The residues were separately redissolved in methanol–water–triethanolamine (90:9:1), centrifuged at 10,000 rpm for 10 min, filtered through a 0.45-μm Millipore membrane and quantitatively transferred into a volumetric flask, adjusted to a final volume (1 mL).

Chemicals and standards

All reagents and solvents used for extraction, isolation and chromatographic separation were either of analytical or HPLC grade (E. Merck Ltd., Mumbai, India), while the reference marker compounds **4H**, its glucoside (**T4**) and **EA** were isolated in high purity (>95%), determined by HPLC coupled with photodiode array detection (PDA) analysis in our laboratory.

Apparatus and chromatographic conditions

A sonicator (Microclean 109, Oscar Ultrasonic, Mumbai, India) was used for sample preparation and degassing of HPLC mobile phases. The 300-MHz NMR (Avance, Bruker, Switzerland) was used to record ^{1}H and ^{13}C NMR with tetramethylsilane (TMS) as internal standard. The compounds were first visualized on TLC plates (silica gel 60F$_{254}$, Merck) under ultraviolet (UV) illumination at 254 and 365 nm and then sprayed with vanillin–sulphuric acid (1:5, w/v) solution in ethanol, followed by heating at 95°C for 5 min. HPLC analysis was performed on a Shimadzu LC-10AD liquid chromatograph equipped with an SPD-M10A VP diode array detector, an SIL-10ADVP auto-injector and CBM-10 interface module. Data were collected and analyzed using a class LC-10 Work Station. A Chromatopak Peerless C18 column (250 × 4.6 mm i.d., 5 μm) was selected for HPLC analysis. The separation was achieved with a gradient program for pump A [0.1% trifluoroacetic acid (TFA) in water] and pump B (methanol) with a linear gradient elution as follows: 75% A (5 min), 75–70% A (20 min), 70–75% A (25 min). The flow rate was 0.6 mL/min throughout the gradient run. Column temperature was maintained at 25 ± 1°C.

Preparation of standard solution

Standard stock solutions of **4H**, its glucoside (**T4**) and **EA** were separately prepared at a concentration of 1.0 mg/mL in methanol. A serial dilution was made for each stock solution at concentrations of 3–15 μg/mL by adding methanol and 20 μL of each was used for plotting the standard curve for **4H**, **T4** and **EA**.

Results and Discussion

Method development: optimization of HPLC conditions

HPLC with UV detection has commonly been employed in the analysis of secondary metabolites in plants. The signal for UV detection is a consequence of chromophoric groups and conjugation in the secondary metabolites. In the present study, method development was based on HPLC–PDA detection. Different types of columns and mobile phase compositions were carefully tested to determine the optimal chromatographic conditions. Better separation and peak resolutions were achieved with a reversed-phase Chromatopak Peerless C18 column (250 × 4.6 mm i.d., 5 μm). As an organic modifier for mobile phase, methanol performed better than acetonitrile; hence, we tested methanol and water in different proportions, but the results were not satisfactory. Furthermore, when we used TFA in the concentration range of 0.1–1.0% as an acid additive in water to improve peak symmetry and response, the results were good. Therefore, optimization of gradient elution was performed with different proportions of methanol and 0.1% TFA in water. A column temperature of 25°C was required to obtain reproducible peaks, because a higher temperature caused poor peak resolution. The preceding series of investigations led us finally arrive at an optimal eluting condition for the simultaneous determination of bioactive compounds **4H**, **T4** and **EA** in *A. baccifera* root and aerial extracts. The results were good and reproducible in one-step gradient. The retention times were 7.3 ± 0.04, 13.2 ± 0.06 and 18.7 ± 0.04 min for **EA**, **T4** and **4H**, respectively. To select the absorption wavelength for detection, UV spectra of the analytes and extract samples were acquired and overlaid. The best response was found at 254 nm for all three analytes.

Because the UV absorption maxima of all three analytes (**4H**, **T4** and **EA**) was 254 nm, chromatograms for their quantitative analyses were recorded at 254 nm. Chromatograms of mixed standards and sample are shown in Figures 2A–C, and revealed good separation for most of the peaks. Chromatographic peaks were identified by comparing their retention times and UV absorption spectra with those acquired for standards analyzed under the same chromatographic conditions. A column performance report for *A. baccifera* plant extract is presented in Table I. As a measure of column performance, the numbers of theoretical plate counts (N) for **EA**, **T4** and **4H** were 5,365, 3,746 and 6,139, respectively.

Method validation

Method validation was performed on parameters such as linearity, limit of detection (LOD) and limit of quantification (LOQ), specificity, precision, accuracy, recovery and robustness, as per International Conference on Harmonization (22) guidelines. All data were evaluated using standard statistical packages for Windows and Graph Pad Prism 4.0 (Graph Pad Software Inc., La Jolla, CA).

System suitability test

System suitability was assessed by six replicate analyses of the analytes. The acceptance criterion was ± 2% for the percent relative standard deviation (%RSD) of peak area and retention time. The tailing factor was also determined and the results are given in Table I.
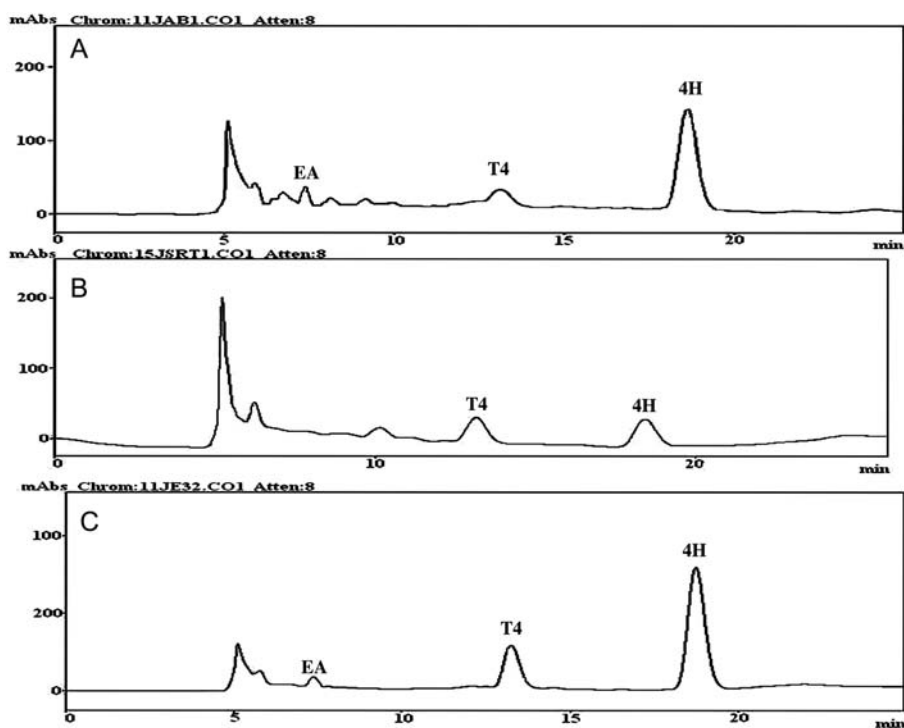

Figure 2. HPLC chromatogram: methanolic extract of *A. baccifera* leaf (A); methanolic extract of *A. baccifera* root (B); artificial mixture of standard compounds **EA**, **T4** and **4H** (C).

Table I
System Suitability Study for the Determination of Bioactive Compounds **4H**, **T4** and **EA** ($n = 6$)

Compound		Retention time (min) (10 µg/mL)	Peak area	Tailing factor	Theoretical plates
EA	Mean	7.3 ± 0.04	257,663 ± 0.26	1.15 ± 0.010	5,365 ± 0.08
	%RSD	0.547	1.009	0.869	0.001
T4	Mean	13.2 ± 0.06	3,525,039 ± 0.38	1.19 ± 0.010	3,746 ± 0.06
	%RSD	0.454	1.078	0.840	0.001
4H	Mean	18.7 ± 0.04	11,246,151 ± 0.12	1.14 ± 0.012	6,139 ± 0.08
	%RSD	0.213	1.067	1.052	0.001

Table II
Linear Regression Parameters for the Separation of Bioactive Compounds **4H**, **T4** and **EA**

Compound	Linear regression parameters ($n = 5$)		
	Slope	y-intercept	r^2
EA	209,752	−657,644	0.999
T4	7,382.6	+280	0.999
4H	24,318	−−3,979.3	0.999

Linearity and sensitivity (LOD and LOQ)

The calibration curves for **4H**, **T4** and **EA** were plotted with five different concentrations from 2 to 10 µg/mL. The detector response was linear. The linearity was assessed by calculating the slope, y-intercepts and coefficient of correlation (r^2) using a least squares regression equation. The detailed descriptions of regression curves are shown in Table II, which shows good linearity ($r^2 = 0.999$) for all three analytes in the examined concentration range. The LOD and LOQ of this method were determined as a signal-to-noise ratio of 3 for LOD and 10 for LOQ. Six replicate injections of the solution gave an RSD of peak area ≤1.07%. The LODs were found to be 1.51, 0.70 and 0.22 µg/mL, whereas LOQ were found to be 5.06, 2.33 and 0.73 µg/mL for **EA**, **T4** and **4H**, respectively.

Selectivity

The selectivity of the method was determined by analysis of standard compounds and samples. The peaks of **4H**, **T4** and **EA** were identified by comparing their retention times in a UV spectrum with those of the standards. The matching UV spectra were 0.9994, 0.9995 and 0.9998, respectively. Spiking of selected samples with the standard compounds was also used to confirm peak identity.

Precision and accuracy

The intra-day and inter-day precision and accuracy determination of bioactive constituents (**4H**, **T4** and **EA**) are given in Table III. The %RSD values for intra-day precision were 0.556−1.006, 0.415−1.324 and 0.586−0.987% for **EA**, **T4** and **4H**, respectively, and inter-day precisions were 0.334−1.496, 0.134−1.556 and 1.346−1.730%, respectively. The low values of %RSD (≤1.73%) reflect the high precision of the method. The recoveries for intra-day accuracy were 99.3−103.0, 99.6−101.5 and 96.6−104.6%, respectively, and those for inter-day accuracy were 98.0−99.6, 99.2−101.3 and 99.0−104.0%, respectively for **EA**, **T4** and **4H**. All percentage recoveries were within 96.6−104.6%, indicating the good accuracy of the method.

Table III
Intra-Day and Inter-Day Precision (%RSD) and Accuracy (% recovery) of Bioactive Compounds **4H**, **T4** and **EA**

Compound	Amount added (µg/mL)	Amount found (µg/mL) ± SD	%RSD	% Recovery	Average % recovery
Intra-day ($n = 6$)					
EA	3	2.98 ± 0.030	1.006	99.30	100.6
	6	6.20 ± 0.036	0.580	103.00	
	9	8.98 ± 0.050	0.556	99.70	
T4	3	3.02 ± 0.040	1.324	100.60	100.56
	6	5.98 ± 0.034	0.568	99.6	
	9	9.14 ± 0.038	0.415	101.5	
4H	3	3.14 ± 0.026	0.828	104.6	100.06
	6	5.80 ± 0.034	0.586	96.6	
	9	8.91 ± 0.088	0.987	99.0	
Inter-day ($n = 6$)					
EA	3	2.94 ± 0.044	1.496	98.0	99.06
	6	5.96 ± 0.064	1.074	99.6	
	9	8.97 ± 0.030	0.334	99.6	
T4	3	3.02 ± 0.047	1.556	100.6	100.36
	6	6.08 ± 0.030	0.493	101.3	
	9	8.93 ± 0.012	0.134	99.2	
4H	3	3.12 ± 0.054	1.730	104.0	100.76
	6	5.94 ± 0.080	1.346	99.0	
	9	8.94 ± 0.121	1.353	99.3	

Table IV
Robustness Testing for Bioactive Constituents **4H**, **T4** and **EA**

Parameter	%RSD of retention time			%RSD of peak area		
	EA	T4	4H	EA	T4	4H
Mobile phase composition	0.454	1.021	0.364	1.450	1.814	0.991
Flow rate	0.824	0.562	0.589	1.842	1.355	1.806
Column temperature	0.618	0.718	0.196	0.982	1.093	1.041

Recovery

The three different concentrations diluted from the stock solution were added to an extract with a known content of **EA**, **T4** and **4H** and the recovery of the respective constituents was calculated. The recovery (R) was calculated as $R = (C_{found} - C_{sample})/C_{added}$, where C_{found} is the concentration in the spiked sample, C_{sample} is the concentration in the sample before spiking and C_{added} is the concentration of added standard. The results of recovery tests were acceptable as the average recoveries of **EA**, **T4** and **4H** were 99.8, 100.46 and 100.41%, respectively (Table III).

Robustness

To evaluate the robustness of the method, the influence of small and deliberate variations of analytical parameters on retention time and peak area of test compounds was studied. The parameters that were taken into consideration were mobile phase composition, flow rate and temperature. Only one parameter was changed at a time, while the others were kept constant. The %RSDs of retention time and peak area counts were calculated for each parameter and were found to be in agreement with the methods (Table IV).

Method applications

The five samples of *A. baccifera* were collected from Lucknow, Faizabad, Gonda, Barabanki and Sitapur districts of Uttar Pradesh, India in such a way that the distance among these

Table V

Percent Content (μg/g, n = 3) of Bioactive Constituents **4H**, **T4** and **EA** in Five Different Leaf and Root Samples of *A. baccifera*

Sample Name*	EA		T4		4H	
	Leaf	Root	Leaf	Root	Leaf	Root
AB-1	0.1982	—	0.019	0.012	0.930	0.591
AB-2	0.1420	—	0.028	0.016	0.842	0.678
AB-3	0.0982	—	0.029	0.015	0.746	0.586
AB-4	0.1240	—	0.038	0.018	0.948	0.781
AB-5	0.1681	—	0.036	0.014	0.780	0.682

*Note: Places of collection: AB-1, Lucknow; AB-2, Gonda; AB-3, Barabanki; AB-4, Faizabad; AB-5, Sitapur

places was not less than 60 kilometers. These samples were quantified for the bioactive constituents (**4H**, **T4** and **EA**) by the developed and validated method. The results showed that all three bioactive markers were present in all leaf samples, but the leaf sample collected from Faizabad showed a maximum amount of marker compounds **4H** and **T4**, while the leaf sample collected from Lucknow showed a maximum amount of **EA**. On the other hand, all of the root samples showed an absence of **EA**, but the root sample collected from Faizabad showed a maximum amount of marker compounds **4H** and **T4** (Table V).

Conclusion

Owing to the bioactive potential, **4H**, **T4** and **EA** were selected as chemical markers of *A. baccifera*. In this study, a simple, gradient RP-HPLC−UV method was developed and validated for the simultaneous determination of individual bioactive molecules in the leaf and root extracts of *A. baccifera*. The experimental conditions, including the mobile phase composition, column temperature and flow rate, were optimized to provide high resolution and reproducible peaks. A flow rate of 0.6 mL/min was found to be appropriate for shortening the run time without compromising the peak resolution. A column temperature of 25°C was required to obtain reproducible peaks, while a higher temperature caused poor peak resolution.

The developed and validated method is suitable for the simultaneous determination of individual bioactive molecules in *A. baccifera* leaf and root extracts with excellent precision, accuracy and linearity. The gradient method is simple, rapid, easy and reproducible, and may be used for routine analysis of these bioactive molecules (**4H**, **T4** and **EA**) in the extracts and formulations of *A. baccifera*.

Acknowledgments

References

1. Huang, M., Yin, H., Zhang, X.; A Chinese medicinal composition for the treatment of female infertility; Chinese patent, 1110585 A; October 25, 1995.

2. Zhang, H.; A composition for the treatment of gastroenteropathy comprising *fructus litseae pungentis*, *herba ammanniae bacciferae*, *massa medicata fermentata* powder; Chinese patent, 1398606 A; February 26, 2003.

3. Meng, G.; Chinese medicinal composition for treatment of spinal disease; Chinese patent, 101417003 A; April 29, 2009.

4. Cao, Y.; Chinese herbal medicine for treating hemorrhoids and preparation thereof; Chinese patent, 101549115 A; October 07, 2009.

5. Cao, J.; Traditional Chinese medicine preparation (oral administration) for treating urethritis; Chinese patent, 101954011 A; January 26, 2011.

6. Zhu, H.; Chinese medicine for treating common cold in summer and preparation method thereof; Chinese patent, 102078584 A; January 06, 2011.

7. Wu, H., Wu, J.; A Chinese medicinal liquid for the treatment of abscess, sore, and itching; Chinese patent, 101524449 A; September 09, 2009.

8. Parekh, J., Chanda, S.; Antibacterial and phytochemical studies on twelve species of Indian medicinal plants; *African Journal of Biomedical Research*, (2007); 10: 175−181.

9. Upadhyay, H.C., Dwivedi, G.R., Darokar, M.P., Chaturvedi, V., Srivastava, S.K.; Bioenhancing and anti-mycobacterial agents from *Ammannia multiflora*; *Planta Medica*, (2012); 78: 79−81.

10. Prasad, K.V., Bharathi, K., Srinivasan, K.K.; Evaluation of *Ammannia baccifera* Linn. for antiurolithic activity in albino rats; *Indian Journal of Experimental Biology*, (1994); 32: 311−313.

11. Thakkar, S.M., Deshmukh, V.K., Saoji, A.N., Duragkar, N.J.; Chemical examination of Ammannia baccifera Linn. (Lythraceae); *Journal of the Indian Chemical Society*, (1986); 63: 619−620.

12. Deeseenthum, S., Tip-Pyang, S., Wattanasirmkit, K., Phuwapraisirisan, P.; Toxic metabolites from *Ammannia baccifera*; *Asian Coordinating Group for Chemistry*, (2000); 12: 47−50.

13. Banzouzi, J.-T., Prado, R., Menan, H., Valentin, A., Roumestan, C., Mallie, M. *et al.*; *In vitro* antiplasmodial activity of extracts of *Alchornea cordifolia* and identification of an active constituent: Ellagic acid; *Journal of Ethnopharmacology*, (2002); 81: 399−401.

14. Srivastava, A., Rao, L.J.M., Shivanandappa, T.; Isolation of ellagic acid from the aqueous extract of the roots of *Decalepis hamiltonii*: Antioxidant activity and cytoprotective effect; *Food Chemistry*, (2007); 103: 224−233.

15. Lin, W.Y., Peng, C.F., Tsai, I.L., Chen, J.J., Cheng, M.J., Chen, I.S.; Antitubercular constituents from the roots of *Engelhardia roxburghiana*; *Planta Medica*, (2005); 71: 171−175.

16. An, T.Y., Hu, L.H., Chen, R.M., Chen, Z.L., Li, J., Shen, Q; Anti-diabetes Agents—I: Tetralone derivatives from *Juglans regia*; *Chinese Chemical Letters*, (2003); 14: 489−490.

17. Fournet, A., Angelo, A.B., Munoz, V., Hocquemiller, R., Roblot, F., Cave, A.; Antileishmanial activity of a tetralone isolated from *Ampelocera edentula*, a Bolivian plant used as a treatment for cutaneous leishmaniasis; *Planta Medica*, (1994); 60: 8−12.

18. Naczk, M., Shahidi, F.; Phenolics in cereals, fruits, vegetables: Occurrence, extraction and analysis; *Journal of Pharmaceutical and Biomedical Analysis*, (2006); 41: 1523−1542.

19. Bala, I., Bhardwaj, V., Hariharan, S., Kumar, M.N.V.R.; Analytical methods for assay of ellagic acid and its solubility studies; *Journal of Pharmaceutical and Biomedical Analysis*, (2006); 40: 206−210.

20. Verma, R.K., Bhartariya, K.G., Gupta, M.M., Kumar, S.; Reverse-phase high performance liquid chromatography of asiaticoside in *Centella asiatica*; *Phytochemical Analysis*, (1999); 10: 191−193.

21. Maurya, A., Verma, R.K., Srivastava, S.K.; Quantitative determination of bioactive alkaloids lysergol and chanoclavine in *Ipomoea muricata* by reversed-phase high-performance liquid chromatography; *Biomedical Chromatography*, November 25, 2011: .

22. International Conference on Harmonization (ICH), Q2A (2005); Validation of Analytical Procedures: Text and Methodology.