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A simple and reliable method for the determination of tetralone-4-O-β-D-glucopyranoside and 4-hydroxy-α-tetralone in three species of *Ammannia* by reversed-phase HPLC

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ABSTRACT

*The species of genus Ammannia have proven medicinal efficacies, being frequently used in traditional Chinese and Indian systems of medicine. The compounds 4-hydroxy-α-tetralone and its glucoside have been identified as the major bioactive constituents in Ammannia species. A rapid, reliable and reproducible reversed-phase HPLC method was developed for the determination of tetralone-4-O-β-D-glucopyranoside (**1**) and 4-hydroxy-α-tetralone (**2**) in three species of Ammannia namely, A. coccinea, A. verticillata and A. tenuis is repoted here. The chromatographic separation of compounds was performed on a Waters Spherisorb ODS2 (250 X 4.6 mm i.d., 10µm) column by one step gradient elution with 0.1% trifluoroacetic acid (TFA) in water and methanol at a flow rate of 0.4 mL/min, a column temperature at 25±2°C and ultra-violet (UV) detection at λ 254 nm. The limit of detection (LOD) and limit of quantification (LOQ) were 0.30 and 0.47 µg/mL for analyte **1** and 1.01 and 1.33 µg/mL for analyte **2,** respectively. Satisfactory results with respect to linearity ($r^2 > 0.999$), repeatability (relative standard deviation, RSDd ≤ 1.23%) and recovery (96.8-100.8%) were obtained with this method. The developed method was successfully applied for the quantification of these two analytes (**1** & **2**) in seven different samples of the investigated three Ammannia species.*

INTRODUCTION

Herbal medicines are being widely used in traditional health care system for thousands of years in many countries like India, China, Korea and Japan. Today, screening of plants as a source of novel chemical entities for drug development is a widely persued research activity allover the world [10, 11]. A large number of bioactive compounds such as digoxin, morphine, taxol, atropine, artemisinin, vincristine and vinblastine etc have been isolated and characterized from plants and are now being used as modern drugs [3, 5]. For the past few decades, global demand for traditional medicines is increasing enormously due to the adverse side effects of modern synthetic drugs [2]. Since a majority of herbal formulations generally contain one or more active chemical consitituent(s) whose quality may often vary with season of harvest, developmental age of the component herb (s) and method of their post-harvest processing, it becomes imperative to develop easy & reliable methods for their qualitative and quantitative analysis for ascertaining quality of their finished products [12.13].

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The plants of genus *Ammannia,* commonly called as 'Red stems', are widely distributed in India; growing mostly in marshy places, at the bank of rivers, rivulets, ponds and in paddy fields [14]. The species of *Ammannia* are prized for their medicinal values and many species are being used in traditional medicines to cure a variety of diseases viz. urethritis, abscess, sore, and itching, spinal disease, human female infertility, gastroenteropathy, rheumatic diseases and all types of cancer [14, 15]. In our earlier studies, the compounds 4-hydroxy-α-tetralone (**2**) and its glucoside (**1**) were identified as bioactive markers constituent in *Ammannia* [14, 15]. The literature search revealed that the 4-hydroxy-α-tetralone (**1**) possess potent anti-tuberculosis [8], anti-diabetic [1] and anti-leishmanial [14] activities. Recently, we have also reported bioenhancing activity of 4-hydroxy-α-tetralone, its glucoside and various semi-synthetic acyl derivatives [14]. We have also reported a HPLC methods for the determination of bioactive components in two species of *Ammannia* namely, *A. multiflora* and *A. baccifera* [16,17], In this communication, a validated HPLC analytical method for the determination of these bioactive components (**1** & **2**) in three other species of this genus namely *A. coccinea, A. verticillata and A. tenuis* is reported. The developed method can be gainfully employed for quality assurance of *Ammannia*- based herbal formulations available in the trade.

MATERIALS AND METHODS

Plant material and sample preparation

The plant samples of *A. coccinea, A. verticillata and A. tenuis* were collected locally from Lucknow district of Uttar Pradesh (India). Taxonomic identification of the plant species was made 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. The voucher specimens of *A. coccinea* (No.-9463), *A. verticillata* (No.-9456) and *A. tenuis* (No. 9485) were deposited in the plant herbarium of CSIR-CIMAP.

Isolation and characterization of marker compounds

The marker compounds **1** & **2** were isolated in high purity as per our earlier report [15]. Briefly, the dried and finally milled plant samples (particle size about 60 mesh, 1.0 g) were extracted through sonication (Microclean 109, Oscar Ultrasonic, Mumbai, India) in methanol for 30 min. The solvent was removed under vacuum at 40^{0}C and the extract was re-dissolved in 1 mL of methanol and centrifuged at 10000 rpm for 10 min, filtered through a 0.45 µm millipore membrane and quantitatively transferred into a volumetric flask and adjusted to a final volume of 1 mL. All the reagents and solvents used for extraction, isolation and chromatographic separation were of analytical or HPLC grade (E. Merck Ltd., Mumbai, India), while the reference marker compounds (**1** & **2**) were isolated in high purity (>95%), determined by HPLC coupled with photodiode array detection (PDA) analysis in our laboratory.

HPLC Conditions

HPLC analysis was performed on a Shimadzu LC-10AD Liquid chromatograph equipped with a SPD-M10A VP Diode array detector, a SIL-10AD VP auto injector and CBM-10 interface module. Data were collected and analyzed using a class LC-10 Work Station. A Waters Spherisorb ODS2 (250 X 4.6 mm i.d., 10µm) column was selected for HPLC analysis. The separation was achieved with a gradient program for pump A (0.1% TFA in water) and pump B (methanol) with a one step gradient elution. The flow rate was 0.4 mL/min throughout the gradient run. Column temperature was maintained at 25±2^{0}C for quantification of marker molecules in the samples. Standard stock solutions of compounds (**1** & **2**) were separately prepared at a concentration of 1.0 mg/mL in methanol. Serial dilutions of these were made at concentrations of 2-10 µg/mL by adding methanol. 20 µL of each was used for plotting the standard curves.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The method developed here was based on

HPLC-PDA detection. Different types of columns and mobile phase compositions were tested in order to determine the optimal chromatographic separation. Optimal separation and peak resolutions were achieved with a reversed phase Waters Spherisorb ODS2 (250 X 4.6 mm i.d., 10µm) column. As an organic modifier for mobile phase, methanol performed better than acetonitrile. Hence, we tested methanol and water in different proportions that gave separation of standards using gradient elution, However, under these conditions the peak resolution was still not satisfactory. Further, when we used TFA in the range of 0.1-1.0% as an acid additive in water to improve peak symmetry and response, the results were better. Therefore, further optimization was carried out with gradient elution of methanol and 0.1% TFA in water with different proportions (Table 1). A column temperature of 25±2^{0}C was required to obtain reproducible peaks while the higher temperature caused poor peak resolution. This finally led us to at an optimal eluting condition for the simultaneous determination of bioactive compounds (**1** & **2**) in the samples (Fig 1&2). The results were good and reproducible in one step gradient. The retention times were 7.7±0.02 and 9.6±0.04 min for analytes **1** and **2** 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 both the analytes (**1** & **2**).

Table 1. Gradient program for the separation of bioactive compounds (1 & 2) in extracts of *Ammannia sp.*

Time (min)	Pump A % (0.1% TFA in water)	Pump B % (Methanol)	Flow rate (mL/min)
0.0	60	40	0.4
15.0	70	30	0.4
20.0	60	40	0.4

Peaks were identified by comparing their retention times and UV absorption spectra with those acquired for standards analyzed under the same chromatographic conditions. Column

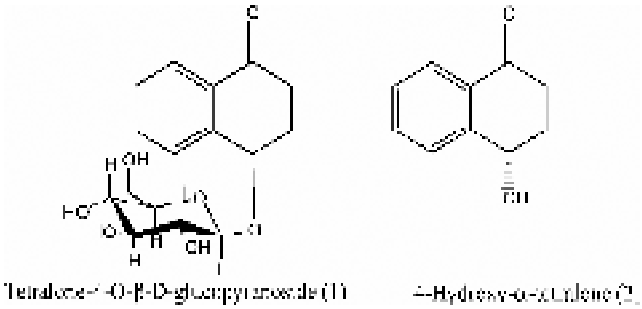

Fig. 1: Structures of the bioactive compounds (1 & 2)

performance report for the plant extracts is presented in Table 2. As a measure of column performance, the number of theoretical plates (N) for compounds **1** and **2** was 3377 and 5982, respectively. The representative chromatograms of standards and extracts are shown in Figure 2. Limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated for quantitative purposes. The samples were analyzed within same day and for consecutive days to assess intra-day and inter-day precision and accuracies. The low values of % RSD (≤ 1.23%) reflect the high precision of the method. Hence, the proposed RP-HPLC method was found precise and accurate. Further, the method was found to be sensitive enough for the analysis of compounds **1** and **2** in the roots, stem and leaf samples of the *A. coccinea, A. verticillata and A. tenuis*.

Method validation

Method validation was performed on parameters such as linearity, LOD and LOQ, specificity, precision, accuracy, recovery and robustness as per International Conference on Harmonization (ICH) guidelines [6]. All the data were evaluated using standard statistical packages for Windows and Graph Pad Prism 4.0 (Graph Pad Software Inc., USA).

System suitability test

System suitability was assessed by six replicate analyses of the analytes. The acceptance criterion was ± 2% for the % RSD of peak area and retention time. The tailing factor was also determined and the results are presented in Table 2.

Table 2. Column performance report for the determination of analytes 1 & 2 (n=6)

Compound		Retention time (min) (10 µg/mL)	Retention factor	Tailing factor	Theoretical plates
1	Mean	7.672 ± 0.02	0.17	1.01 ± 0.0142	3377 ± 0.00
	%RSD	0.52	0.38	0.92	0.006
2	Mean	9.562 ± 0.04	0.46	1.15 ± 0.0243	5982 ± 0.00
	%RSD	0.85	0.42	0.92	0.004

Linearity and sensitivity

The calibration curves for **1** and **2** were plotted with five different concentrations at 2-10 µg/mL. The detail descriptions of regression curves are depicted in Table 3. The linearity (r^2=0.999 for both analytes) was observed within the tested concentration range. The LOD and LOQ were 0.30 and 0.47 µg/mL for **1** and 1.01 and 1.33 µg/mL for **2** respectively.

Table 3. Linearity and sensitivity parameters for the determination of analytes 1 & 2

Parameter		Analytes	
		1	2
Retention time (min± SD)		7.7±0.02	9.6±0.04
Linear regression parameters			
	Slope	1921	4029
	y-intercept	786	333
	r^2	0.999	0.999
Limit of detection (LOD)		0.30	1.01
Limit of quantification (LOQ)		0.47	1.33

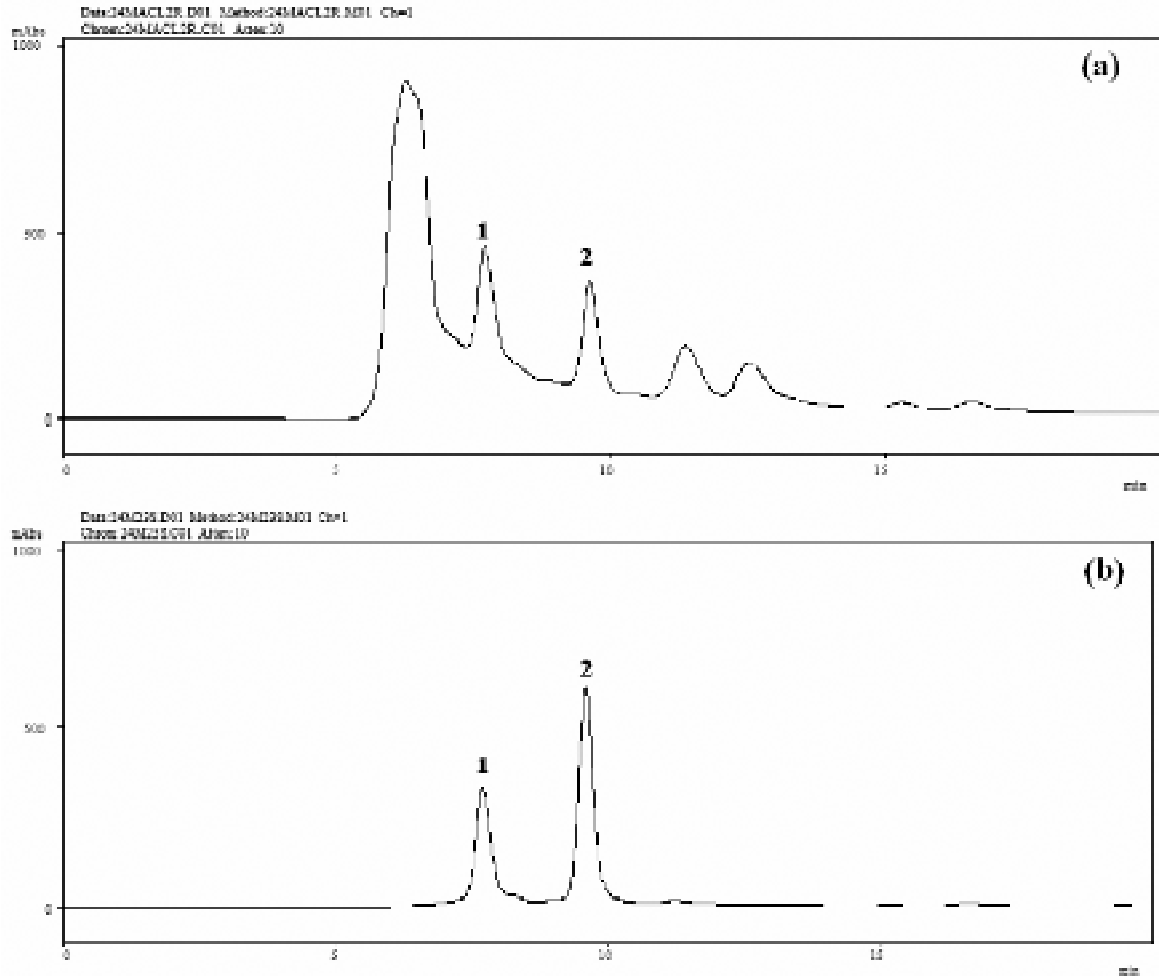

Fig. 2: HPLC chromatogram (a) Methanolic extract of *A. coccinea* leaf (b) mixture of standard compounds 1 & 2

Precision and Accuracy

The accuracy of the assay was measured in terms of recovery of analytes **1** and **2.** The results of recovery of the tests were acceptable as the percentage recoveries of **1** and **2** for intra-day accuracies were 98.4-103.2 and 98.4– 100.8% and those for inter-day accuracies were 99.2-100.8 and 96.8–98.5% respectively (Table 4). The intra-day and inter-day precision of the method were assessed by measurement of % RSD of the results of the recovery for compounds **1** and **2**. The % RSD values for intra-day precision were 0.402-0.775 and 0.010-0.398 for **1** and **2** respectively, and those for inter-day precision were 0.403-0.793 and 0.405-1.230 respectively. The low values of % RSD (≤ 1.23%) reflected the high precision of the developed method.

Table 4. Intra and inter day precision (% RSD) and accuracy (% recovery) parameters of analytes 1 & 2

Analyte	Amount added (µg/mL)	Amount found (µg/mL) ±SD	% RSD	% Recovery	Average recovery
Intra-day (n=6)					
1	2.5	2.58±0.02	0.775	103.20	100.30
	5.0	4.92±0.02	0.406	98.40	
	7.5	7.45±0.03	0.402	99.30	
2	2.5	2.46±0.04	0.010	98.40	99.86
	5.0	5.02±0.02	0.398	100.40	
	7.5	7.56±0.03	0.396	100.80	
Inter-day (n=6)					
1	2.5	2.52±0.02	0.793	100.80	99.90
	5.0	4.96±0.02	0.403	99.20	
	7.5	7.48±0.04	0.534	99.70	
2	2.5	2.42±0.03	1.230	96.80	97.77
	5.0	4.90±0.02	0.408	98.00	
	7.5	7.39±0.03	0.405	98.50	

Method applications

The developed and validated method was applied for the quantification of two analytes (**1** & **2**) in six different samples corresponding to roots, stem and leaves of *A. coccinea* and *A. verticillata*; and a sample of *A. tenuis* (whole plant). The assay results of different samples is summarized in Table 5. Careful analysis of the results revealed that the aerial part (stem/leaf) contains more amounts of the analytes **1** and **2** than the roots.

Table 5. Percent content (w/w) of analytes 1 & 2 in different extracts of *Ammannia* species

Plant name	Plant part	Amount (w/w) in methanolic extract	
		1	**2**
A. coccinea	Root	0.0070±0.002	0.4529±0.002
	Stem	0.0488±0.004	0.5723±0.003
	Leaf	0.0426±0.002	0.3461±0.004
A. verticillata	Root	0.0103±0.003	0.0835±0.003
	Stem	0.0428±0.006	0.1390±0.006
	Leaf	0.0621±0.004	0.1482±0.008
A. tenuis	whole plant	0.0408±0.004	0.2320±0.002

CONCLUSION

The quantification of **1** and **2** in *A. coccinea*, *A. verticillata* and *A. tenuis* is being reported here for the first time. The developed RP-HPLC method is simple, precise, selective and sensitive with acceptable precision, accuracy, and linearity within the test ranges for both the analytes. The total run time was less than 15 min. The developed method may be gainfully employed for the quality control of finished herbal products containing these *Ammannia* species.

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