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*Research Article*

# A Validated LC Method for Separation and Determination of Tetralone-4-O-$\beta$-D-Glucopyranoside and 4-Hydroxy-$\alpha$-Tetralone in *Ammannia multiflora*

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A rapid, sensitive, and reproducible RP-HPLC method was developed for the determination of two constituents in *Ammannia multiflora*, namely, tetralone-4-O-$\beta$-D-glucopyranoside (**1**) and 4-hydroxy-$\alpha$-tetralone (**2**). The samples were separated on a Spherisorb ODS2 column (250×4.6 mm, i.d., 10 $\mu$m) and binary elution of water and methanol (2 : 3) with flow rate of 0.8 mL/min at $\lambda$ 254 nm. The LOD and LOQ were found 0.05 and 0.18 $\mu$g/mL for compound **1** and 0.06 and 0.18 $\mu$g/mL for compound **2**, respectively. All calibration curves showed good linearity ($r^2 > 0.999$) within test ranges for both the analytes. The RSD values for intra-and inter-day precisions were less than 1.1%. The successful application of the developed method on five different samples revealed an average 0.0206% and 0.7636% (w/w) of compounds **1** and **2**, respectively in *A. multiflora* indicating that the developed LC assay method may be readily utilized as a quality control method for the plant.

## 1. Introduction

The genus Ammannia (Family Lythraceae) is commonly called as "red stems," and species of this genus are nearly cosmopolitan in distribution including India. One species of this genus, *Ammannia baccifera* is frequently used in traditional Chinese medicine to cure human female infertility, gastroenteropathy, as antipyretic, antidiuretic, antimicrobial, antirheumatic, anticancer, rubefacient, and as an external remedy for ringworm and skin diseases [1]. *Ammannia multiflora* grows in marshy places along the banks of rivers and rivulets and generally found in cultivated paddy fields. In Tanzania, the juice of fresh leaves of *A. multiflora* is used to treat sight problems in human adults, caused by filaria [2]. The preliminary phytochemical analysis of *A. multiflora* showed presence of glycosides, flavonoids, carbohydrates, steroids, phenols, and absence of alkaloids [3]. The GC-MS analysis of hexane and chloroform fraction led to identification of fourteen constituents, many of them are well-known bioactive phytomolecules [3]. Recently, we isolated and characterized compounds **1** and **2** from *A. multiflora* and reported bioenhancing activity of **2** and its various semisynthetic acyl and aryl derivatives [1]. Compound **2** has shown potent antituberculosis [4], antidiabetic [5], and antileishmanial [6] activities in earlier studies. Until now there has been no quantitative method for the analysis of *A. multiflora*, hence the current study was aimed at developing and validating a simple, rapid, and sensitive reverse phase HPLC method for the quantification of these two constituents in order to have quality control in *A. multiflora*. The present work is part of a series of efforts towards developing analytical methods for plant drug analysis.

## 2. Experimental

*2.1. Plant Material and Sample Preparation.* The samples of *A. multiflora* were collected from the Lucknow, Faizabad, Gorakhpur, Barabanki, and Sitapur districts of Uttar

Table 1: Solvent system for the separation of analytes **1** and **2** from the MeOH extract of *A. multiflora*.

| Time (min) | Water conc. (%) (pump A) | Methanol conc. (%) (pump B) |
|---|---|---|
| 0.00 | 40 | 60 |
| 15.00 | 70 | 30 |
| 20.00 | 40 | 60 |

Pradesh, India in the month of November 2009 and identified by Dr. D. C. Saini, Scientist, Birbal Sahni Institute of Palaeobotany (BSIP), Lucknow and Dr. S. C. Singh, Scientist, Botany Division, Central Institute of Medicinal & Aromatic Plants (CIMAP), Lucknow, India. A voucher specimen (no.−9455) has been deposited at the CIMAP herbarium.

The dried and finally milled plant material (particle size about 60 mesh, 0.1 g) of *A. multiflora* (whole plant) was extracted by sonication for 30 min using methanol as solvent. The solvent was removed under vacuum at 40°C, and the extract was redissolved in 1 mL of methanol and centrifuged at 10000 rpm for 10 min, filtered through a 0.45 $\mu$m Millipore membrane (Millipore, Billerica, MA, USA).

*2.2. Chemicals and Standards.* The solvents used for extraction were of analytical grade, while the methanol and water used for chromatographic separation were of HPLC grade (all purchased from E. Merck Ltd., Mumbai, India). The reference marker compounds **1** and **2** were isolated in high purity (98.7%) in our laboratory following previously described procedures [1] (see details in supporting information). The purity of isolated compounds was determined by HPLC using area normalization method. Before use in HPLC, the solvents were filtered through a 0.45 $\mu$m Millipore membrane. The samples and mobile phases were degassed by sonicator.

*2.3. Apparatus and Chromatographic Conditions.* HPLC analysis was performed on a Shimadzu LC-10AD Liquid chromatograph equipped with a SPD-M10A VP Diode array detector, a SIL-10ADVP autoinjector and CBM-10 interface module. Data were collected and analyzed using a class LC-10 Work Station. A prepacked Waters Spherisorb ODS2 (250 × 4.6 mm, i.d., 10 $\mu$m) column was selected for HPLC analysis. The separation was achieved with an isocratic program for pump A (water) and pump B (methanol) (Table 1). The injection volume was 20 $\mu$L, and flow rate was 0.8 mL/min throughout the run. Column temperature was maintained at 25 ± 1°C. The data acquisition was performed in the range of 200–400 nm, and quantification was performed at 254 nm. Sonicator (Microclean 109, Oscar Ultrasonic, Mumbai, India) was used for sample preparation. The 300 MHz NMR (Avance, Bruker, Switzerland) was used to record $^{1}$H and $^{13}$C NMR with tetramethylsilane (TMS) as internal standard. Hyphenated LC-PDA-MS (Prominence LC and mass MS-2010EV, Shimadzu, Japan) was used for mass spectra.

*2.4. Preparation of Standard Solutions.* Standard stock solutions of compounds **1** and **2** were prepared as 1.0 mg/mL in methanol. A serial dilution was made for each stock solution at concentrations of 2–10 $\mu$g/mL by adding methanol, and 20 $\mu$L of each was used for plotting the standard curve for **1** and **2**, respectively.

## 3. Results and Discussion

*3.1. Extraction Method.* In order to obtain satisfactory extraction efficiency, extraction method and extraction time were investigated. Initially, two samples of 0.1 g each were separately extracted with 10 mL of methanol at 50°C for 30 min; one by refluxing on water bath while the other by ultrasonication. The w/w% yield of compounds **1** and **2** was 0.014 and 0.702 in refluxing and 0.020 and 0.751 in ultrasonication. As ultrasonication gave better yields, extracting time optimization was studied for 15, 30, 45, and 60 min, respectively. The results showed that the targeted compounds were completely extracted in 30 min. Hence, 30 min ultrasonic extraction with methanol at 50°C was considered as optimal extracting condition.

*3.2. Optimization of Chromatographic Conditions.* Since HPLC with UV detection is the most popular method for analysis of bioactive compounds, method development in the present study was based on HPLC coupled with photodiode array detector (PDA) [7]. Different types of columns and mobile phase compositions were carefully tested in order to determine the optimal chromatographic conditions. It was found that better separation and peak shapes were achieved with Waters Spherisorb ODS2 column (250 × 4.6 mm, i.d., 10 $\mu$m). For the baseline separation first we tried acetonitrile and water in different proportions, but the results were not satisfactory. Hence we switched to water and methanol, which gave us better separations. The optimized programme for pump A (water) and pump B (methanol) was carried out, and the results were good and reproducible in water-methanol (2 : 3). The retention times were 4.1 ± 0.02 and 5.2 ± 0.04 min for **1** and **2**, respectively. The absorption maxima for both analytes were found at 254 nm. Peaks were identified by comparing their retention times and UV absorption spectra with those acquired for standards analyzed under the same chromatographic conditions. Column performance report for *A. multiflora* plant extract is presented in Table 2. As a measure of column performance, the number of theoretical plate counts ($N$) for compounds **1** and **2** was 2053 and 3214, respectively. The representative chromatograms of standards and *A. multiflora* extract are shown in Figure 1. LOD, LOQ, accuracy, and precision were evaluated for quantitative purposes. The samples were analyzed within same day and for consecutive days to assess intraday and interday precision and accuracies. The low values of % RSD (<1.1%) reflect the high precision of the method. Hence, the proposed RP-HPLC method is found to be precise and accurate. Further, the method is sensitive enough for the analysis of compounds **1** and **2** in *A. multiflora*.

Table 2: Column performance report for the determination of analytes **1** and **2** ($n = 5$).

| Analyte | Retention time (min) | Retention factor | Tailing factor | Theoretical plates |
|---|---|---|---|---|
| **1** | | | | |
| Mean | 4.1 ± 0.02 | 3.13 | 1.58 ± 0.0152 | 2053 ± 0.08 |
| % RSD | 0.48 | 0.31 | 0.96 | 0.003 |
| **2** | | | | |
| Mean | 5.2 ± 0.04 | 4.25 | 1.28 ± 0.0142 | 3214 ± 0.07 |
| % RSD | 0.76 | 0.35 | 0.95 | 0.002 |

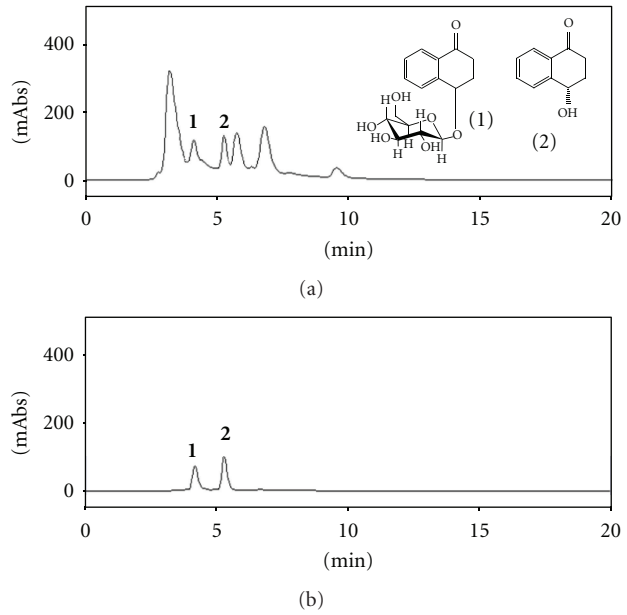


Figure 1: HPLC chromatogram. (a) methanolic extract of *A. multiflora*. (b) artificial mixture of **1** and **2**.

*3.3. Method Validation.* Method validation was performed on the parameters such as linearity, LOD and LOQ, specificity, precision, accuracy, and recovery as per ICH [8] guidelines. All data were evaluated using standard statistical packages for Windows and Graph Pad Prism 4.0 (Graph Pad Software, Inc., USA).

*3.3.1. System Suitability Test.* System suitability was assessed by five replicate analyses of the analytes at a concentration of 10 $\mu$g/mL. The acceptance criterion was ±2% for the percentage relative standard deviation (% RSD) of peak area and retention time. The retention and tailing factors were also determined and the results are given in Table 2.

*3.3.2. Linearity.* The calibration curves for **1** and **2** were plotted with five different concentrations at 2–10 $\mu$g/mL. The detector response was linear, and the linearity was assessed by calculating the slope, $Y$-intercepts and coefficient of correlation ($r^2$) using least squares regression equation. The detail descriptions of regression curves are depicted in Table 3. Good linearity ($r^2 = 0.999$ for both analytes) was observed within the examined concentration range. A statistical residual plot analysis also demonstrated that residuals were randomly distributed around the zero value. This confirms that the model choice is linear.

Table 3: Linearity and sensitivity parameters for determination of analytes **1** and **2**.

| Parameter | **1** | **2** |
|---|---|---|
| Retention time (min ± SD) | 4.1 ± 0.02 | 5.2 ± 0.04 |
| Linear regression parameters | | |
| Slope | 1612.4 | 1605.6 |
| $Y$-intercept | 300.0 | 325.6 |
| $r^2$ | 0.999 | 0.999 |
| LOD ($\mu$g/mL) | 0.05 | 0.06 |
| LOQ ($\mu$g/mL) | 0.18 | 0.18 |

*3.3.3. LOD and LOQ.* The limit of detection (LOD) and limit of quantification (LOQ) of this method were determined as S/N ratio 3 for LOD and 10 fold for LOQ. The low values of LOD and LOQ, that is, 0.05 and 0.18 $\mu$g/mL for **1** and 0.06 and 0.18 $\mu$g/mL for **2**, respectively suggest the high detection capability of the method.

*3.3.4. Precision and Accuracy.* The accuracy of the assay was measured in terms of recovery of analytes **1** and **2**. The three different concentrations diluted from the stock solution were added to an extract with a known content of compounds **1** and **2**, and the recovery of respective constituents was calculated. The recovery ($R$) was calculated as $R = (C_{\text{found}} - C_{\text{sample}})/C_{\text{added}}$, where $C_{\text{found}}$ is the concentration in spiked sample and $C_{\text{sample}}$ is the concentration in the sample prior to spiking, and $C_{\text{added}}$ is the concentration of added standard. The results of recovery of the tests were acceptable as the percentage recoveries of compounds **1** and **2** for intraday accuracies were 98.40–99.60 and 99.40–100.40%, respectively, and those for interday accuracies were 98.2–101.6 and 98.40–100.2%, respectively, (Table 4). All percentage recoveries were within 98.2–101.6% indicating the good accuracy of the method.

The intraday and interday precision of the method were assessed by measurement of relative standard deviation (% RSD) of the results of the recovery for compounds **1** and **2** (Table 4). The % RSD values for intraday precision were 0.214–0.812 and 0.406–1.015 for **1** and **2**, respectively, and those for interday precision were 0.484–0.590 and 0.548–0.853, respectively. The low values of % RSD (<1.1%) reflect the high precision of the method.

*3.3.5. Assay of Compounds **1** and **2** in A. multiflora.* The developed and validated method was applied for the quantification of two compounds (**1** and **2**) in five different samples of *A. multiflora* collected from Lucknow, Faizabad, Gorakhpur, Barabanki, and Sitapur districts of Uttar Pradesh, India. The sample collected from Lucknow was found to contain

Table 4: Intra-and inter-day precision and accuracy parameters of analytes **1** and **2** ($n = 6$).

| Analyte | Amount added ($\mu$g/mL) | Amount found ($\mu$g/mL) | % RSD | % Recovery |
|---|---|---|---|---|
| | | Intraday | | |
| **1** | 5 | 4.92 ± 0.040 | 0.812 | 98.40 |
| | 10 | 9.96 ± 0.026 | 0.260 | 99.60 |
| | 15 | 14.94 ± 0.032 | 0.214 | 99.60 |
| **2** | 5 | 5.02 ± 0.051 | 1.015 | 100.40 |
| | 10 | 9.94 ± 0.094 | 0.945 | 99.40 |
| | 15 | 15.02 ± 0.061 | 0.406 | 100.13 |
| | | Interday | | |
| **1** | 5 | 5.08 ± 0.030 | 0.590 | 101.6 |
| | 10 | 9.82 ± 0.048 | 0.488 | 98.2 |
| | 15 | 14.85 ± 0.072 | 0.484 | 99.0 |
| **2** | 5 | 4.92 ± 0.042 | 0.853 | 98.40 |
| | 10 | 10.02 ± 0.064 | 0.638 | 100.2 |
| | 15 | 14.96 ± 0.082 | 0.548 | 99.7 |

Table 5: Assay of analytes **1** and **2** in methanolic extracts of *A. multiflora*.

| Sample name | Amount (% w/w) in methanolic extracts | |
|---|---|---|
| | **1** | **2** |
| Lucknow | 0.024 ± 0.003 | 0.823 ± 0.002 |
| Faizabad | 0.019 ± 0.002 | 0.648 ± 0.006 |
| Gorakhpur | 0.021 ± 0.003 | 0.813 ± 0.003 |
| Barabanki | 0.023 ± 0.006 | 0.716 ± 0.007 |
| Sitapur | 0.016 ± 0.002 | 0.818 ± 0.004 |

maximum amount of marker compounds **1** and **2**. The assay results of different samples have been summarized in Table 5.

## 4. Conclusions

A new RP-HPLC method has been developed for the simultaneous determination of two compounds: tetralone-4-O-$\beta$-D-glucopyranoside (**1**) and 4-hydroxy-$\alpha$-tetralone (**2**) in *A. multiflora*. The method is simple, precise, selective, and sensitive having acceptable precision, accuracy, and linearity ($r^2 > 0.999$) within the test ranges for both the analytes. The quantification of compounds **1** and **2** in *A. multiflora* is being reported here for the first time. Due to simple extraction procedure, high precision, accuracy, and short run time of 6 min, the method may be of immense application in the screening of raw materials as well as in the quality control of finished herbal products of *A. multiflora*. The extraction and isolation procedures of marker compounds (**1** and **2**) including their $^1$H, $^{13}$C NMR, and mass spectroscopic data are accessible as Supplementary Material available online at doi:10.1155/2012/162302.

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