

Determination of Withaferin–A in Two *Withania* Species by RP-HPLC Method

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Withania is a small shrub possessing medicinal value commonly found in the Indian subcontinent. Quantitative determination of withaferin-A, was undertaken to provide an easy and simple analytical method, which can be used as a routine quality control method. RP-HPLC was performed using acetonitrile and water as mobile phase. The detection and quantification was performed at a wavelength of 225 nm. Linearity of detector response for withaferin-A was between the concentrations 0.005% to 0.02%. The correlation coefficient obtained for the linearity was

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0.993. The recovery value of standard withaferin-A was 94.4%. Low value of standard deviation and coefficient of variation, are indicative of high precision of the method. The system was successfully used to investigate the marker compound in different plant parts of *W. coagulans* and *W. somnifera*.

Withania (Solanaceae), is a small shrub distributed in east of the Mediterranean region, extending to South Asia. Two species, *Withania coagulans* Dunal and *Withania somnifera* Dunal, are found in India. In India, *W. coagulans* and *W. somnifera* are known by the same names in most of the regional languages. In the market, no distinction is made between the berries obtained from *W. coagulans* and *W. somnifera*. Both the species have identical macroscopical features of roots, such as smooth and whitish surface, and short and starchy fracture. The roots of the *W. coagulans* are however, characterized by the isolated groups of non lignified cells in the xylem tissues, calcium oxalate crystals in the phelloderm cells, and triseriate medullary rays¹.

W. somnifera is a valuable herb in Ayurvedic medicine, and as such, was used and cultivated for centuries in India. Roots, leaves and preparations thereof, are traditionally used as tonic, hypnotic, sedative, and diuretic. The biological activities of withanolides, especially of the dominant withaferin-A have been studied extensively. Notable activities reported for this compound, include antiinflammatory, anticonvulsive, antitumor and antioxidant properties²⁻⁵. Claiming adaptogenic effects, aswagandha products are available throughout the United States of America as dietary supplements^{6,7}. *W. coagulans* leaves are used in Pakistan as a vegetable, and as a fodder for camels and sheep. They are also employed as a febrifuge. The leaves are chewed for cleaning teeth, and the smoke of the plant is inhaled for relief in toothache¹.

Withanolides are ergostane type of steroids with atoms C-22 and C-26, bridged by a δ -lactone functionality, and an oxidized C-1 position. These compounds are specific for the solanaceae family, and in particular, for the genus *Withania*, and thus they are used as marker compounds⁸. The biological activities of withanolides, especially of the dominant withaferin-A has been studied extensively and reported from the roots and leaves of both the species of *Withania*⁹. The number of analytical reports for the determination of withanolides is comparatively small. Besides a TLC method for the quantification of withaferin A¹⁰, a few HPLC methods are described in literature. Most of them showed disadvantages, as either the acetylation of withaferin-A is required prior to analysis¹¹, the separation time is long¹² or the compounds are not

baseline separated and elute, more or less with the injection peak¹³.

A part of our efforts, was to develop a HPLC method suitable for direct determination of withaferin-A in *W. coagulans* and *W. somnifera*, and to investigate the distribution of this compound in different plant parts.

Authenticated *Withania coagulans* and *Withania somnifera* plant materials were collected from Narsapur forest, Medak Dist., Andhra Pradesh, and botanically identified. The plants were shade dried, and leaves and the roots of both the species, were used for HPLC analysis. Standard withaferin-A was procured from the Regional Research Laboratory, Jammu Tawi.

The 4% alcoholic extracts of root, and 0.4% alcoholic extracts of leaf of both the species of *Withania*, were prepared by soaking the respective plant material separately for 18 h, in absolute alcohol. The extracts were filtered through Whatmann filter paper No.1, using high-pressure vacuum pump.

One milligram of standard withaferin-A, was dissolved in 5 ml of alcohol. Three additional calibration levels were prepared by diluting this solution with alcohol, to obtain 0.005%, 0.01%, 0.015% and 0.02% concentrations, for studying the linearity. The precision of the method was also studied, by injecting a sample solution of concentration 0.02% five times, and finding out the standard deviation and coefficient of variation.

A gradient HPLC (Shimadzu HPLC Class VP series) with two LC- 10 AT VP pumps (Shimadzu), variable wave length programmable photo diode array detector SPD-M10A VP (Shimadzu), CTO-10AS VP column oven (Shimadzu), SCL-10A VP system controller (Shimadzu), and reverse phase Luna 5 μ C₁₈ (2) Phenomenex column (250 mm \times 4.6 mm), was used. The HPLC system was equipped with Class VP series version 6.1 software (Shimadzu). The mobile phase components acetonitrile: water were filtered through 0.2 μ membrane filter before use, and were pumped from the solvent reservoir at a flow rate of 1ml/min, which yielded column backup pressure of 135-145 kgf/cm². The initial B-pump concentration was 50%. The column temperature was

maintained at 27°. Rheodyne syringe (Model 7202, Hamilton) was used for injecting 20 µl of respective samples.

Standard withaferin-A solutions of 0.005%, 0.01%, 0.015% and 0.02% concentrations, were analyzed for studying the linearity, and the area count obtained for these solutions are presented in Table 1. Withaferin-A showed good linearity in the concentration range of 0.005% - 0.02%, with a correlation coefficient of 0.993. The precision of the method was also studied by injecting a single sample solution five times (Table 2), and finding out the standard deviation and coefficient of variation. The standard deviation and coefficient of variation were found to be 0.526 and 0.858. Low value of standard deviation and coefficient of variation are indicative of high precision of the method.

The HPLC chromatogram of standard withaferin-A at an optimum wavelength of 225 nm, showed a mean area (Table 2) of 6127383.6, at a mean retention time of 5.883min (Fig. 1). The recovery value of standard withaferin-A was 94.4%. The HPLC chromatogram of leaf of *W. coagulans* corresponding to standard withaferin-A was showed at a retention time of 5.717 min, with an area of 2816990 at a wavelength of 225 nm (Fig. 2). The HPLC chromatogram of root of *W. coagulans* corresponding to standard withaferin-A was showed at a retention time of 5.6 min, with an area of 936833 at a wavelength of 225 nm (Fig. 3). The HPLC chromatogram of leaf of *W. somnifera* corresponding to standard withaferin-A was showed at a retention time of 5.771 min, with an area of 1384751 at a wavelength of 225 nm (Fig. 4). The HPLC chromatogram of root of *W. somnifera* corresponding to standard withaferin-A was showed at a retention time of

5.451 min, with an area of 537152 at a wavelength of 225 nm (Fig. 5). The variation in retention time of peak of withaferin-A in chromatograms of *W. somnifera* and *W. coagulans*, may be due to the presence of other chemical constituents. The quantitative evaluation of withaferin-A in leaf and root of *W. coagulans*, was 2.299% and 0.076%, and that of *W. somnifera* was 1.13% and 0.044%, respectively. High content of withaferin-A in leaf than root was reported from *W. somnifera*⁷.

The proposed method can be used to standardize *Withania species* on the basis of withaferin-A as a

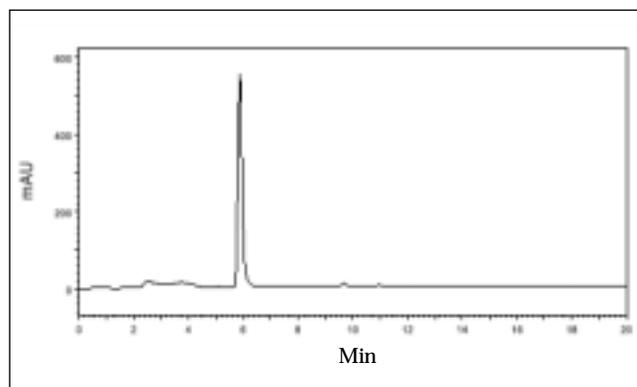


Fig. 1: HPLC chromatogram of standard withaferin-A

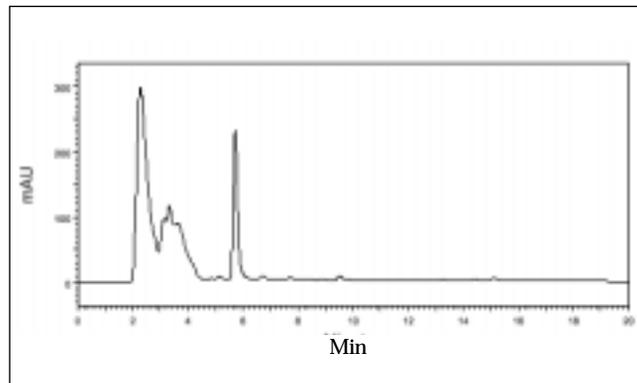


Fig. 2: HPLC chromatogram of *W. coagulans* leaf

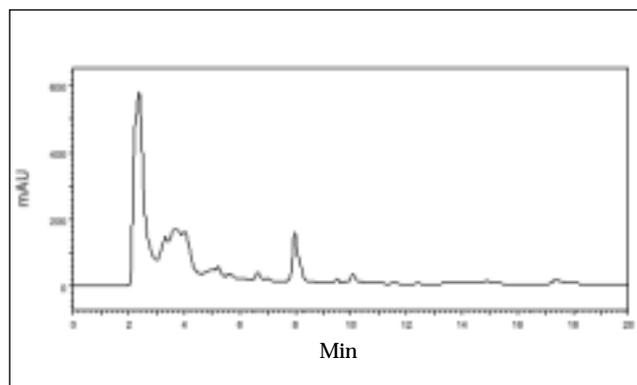


Fig. 3: HPLC chromatogram of *W. coagulans* root

TABLE 1: AREA COUNTS FOR STANDARD WITHAFERIN-A

Concentration of withaferin-A (%)	Area counts
0.005	1677232
0.01	3208899
0.015	4487510
0.02	6205475

TABLE 2: DETERMINATION OF STANDARD DEVIATION AND COEFFICIENT OF VARIATION FOR STANDARD WITHAFERIN-A

Concentration of withaferin-A (%)	Area counts
0.02	6205475
0.02	6126884
0.02	6058190
0.02	6132079
0.02	6114290

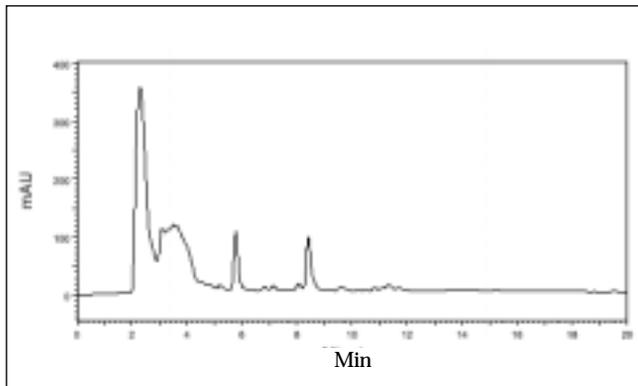


Fig. 4: HPLC chromatogram of *W. somnifera* leaf

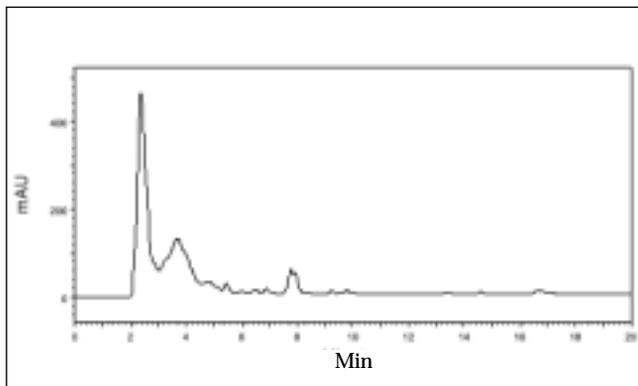


Fig. 5: HPLC chromatogram of *W. somnifera* root

marker compound, and is helpful for scientific as well as commercial applications.

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Accepted 19 March 2006

Revised 4 June 2005

Received 2 December 2004

Indian J. Pharm. Sci., 2006, 68 (2): 253-256