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Research Article

Development and Validation of High Performance Liquid Chromatography Method for Simultaneous Estimation of Flavonoid Glycosides in *Withania somnifera* Aerial Parts

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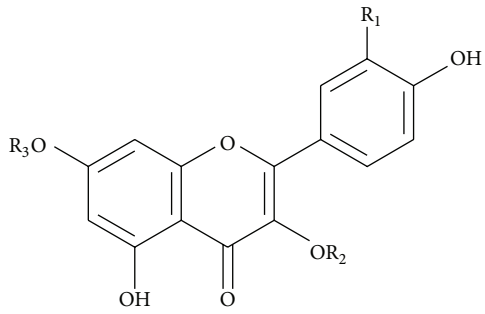
Withania somnifera (L.) Dunal (Solanaceae) commonly known as ashwagandha, is an important plant in Ayurveda and is believed to increase longevity and vitality. The root is considered to be the medicinally important part of the plant as per classical texts and accordingly is the subject of most Pharmacopeial monographs. The aerial parts, being less expensive, are sometimes mixed with roots to prepare "standardized" extracts of *W. somnifera*, and in cases with false declaration of plant part used as roots on the certificate of analysis. The present study described a new, simple, accurate, and precise HPLC method for the simultaneous determination of flavonoid glycosides as unique constituents of the aerial parts, being absent in roots of the plant. The RSD for intra- and interday analyses was less than 2.5% and the recovery was 90–108%. The method was used to analyze samples of roots and aerial parts of the plant collected from India and Egypt. The samples of commercially available extracts of *W. somnifera* were also analyzed and many samples were found to contain flavonoid glycosides indicating a possible undeclared use of aerial parts in the extracts derived from roots in commercial practice.

1. Introduction

Withania somnifera (L.) Dunal (family: Solanaceae), commonly known as ashwagandha or winter cherry, is considered one of the most important medicinal plants in Indian traditional systems of medicine [1–3]. It is a shrubby plant cultivated in India and is primarily used as a rejuvenator and energizer; it grows in parts of East Asia and Africa [4, 5]. The plant has been reported to possess diverse biological activities which include anticancer, antistress, anti-inflammatory [6, 7], antibiotic, anticonvulsant, central nervous system depressant, hepatoprotective, immunomodulatory [8, 9], and insect antifeedant activities.

The chemistry of *W. somnifera* has been extensively studied and over 12 alkaloids, 35 withanolides, several sitoindosides, and flavonoids have been isolated from different parts of the plant [10, 11]. Withanolides, the steroidal lactones with a C28 steroidal nucleus and C9 side chain, containing a six-member lactone ring, are thought to contribute to the bioactivity of the plant. Interestingly, withanolides are present in both the roots as well as in the aerial parts [12, 13]. HPLC and HPTLC-based methods have been reported in the literature for quantitative determination of withanolides in *W. somnifera* [14–21].

The plant has been categorized under "medicinal plant species in high trade sourced largely from cultivation" by the National Medicinal Plants Board, India [22]. The root is considered to be the medicinally important part of the plant as per classical texts and accordingly find place in Pharmacopoeias and in trade. Monographs on roots and



	R_1	R_2	R_3
1	OH	gal(1-6)rha	glc
2	OH	glc(1-6)rha	glc
3	H	gal(1-6)rha	glc

Figure 1: Chemical structure of three flavonoid glycosides of *W. somnifera* leaves.

root extracts of *W. somnifera* now form part of United States Pharmacopoeia (USP), British Pharmacopoeia (BP), Indian Pharmacopoeia (IP), and The Ayurvedic Pharmacopoeia of India (API). Withanolides, have been utilized as the "marker constituents" in Pharmacopoeial monographs. Most of the marketed products derived from the roots of the plant are now being "standardized" based on the content of withanolides. With increased global demand for the roots of *W. somnifera*, cost of the latter has increased considerably. Since the aerial parts are more affordable and also contain withanolides, there has been a tendency to use the aerial parts to achieve "standardization" based on the content of withanolides. Additionally, extracts derived from the aerial parts show similar qualitative chromatographic fingerprints as roots when analyzed for withanolides. This situation has led to availability of some "standardized" products containing *W. somnifera* with misleading declaration on the plant part used for preparation of extract. Availability of a specific analytical method for detection of aerial parts in the roots extract has thus become important.

The present study was designed to develop a new HPLC method which is simple, rapid, reproducible, and economical for simultaneous estimation of three flavonoid glycosides, namely, quercetin 3-*O*-robinobioside-7-*O*-glucoside (**1**), quercetin 3-*O*-rutinoside-7-*O*-glucoside (**2**), and kaempferol 3-*O*-robinobioside-7-*O*-glucoside (**3**), from the aerial parts of *W. somnifera* (Figure 1). These constituents were found to be absent in the root and were analyzed using a newly developed and validated HPLC method. The method described herewith is intended for detection of aerial parts in extracts and products derived from *W. somnifera* roots.

2. Materials and Methods

2.1. Plant Materials. Aerial parts and roots of *W. somnifera* (L.) Dunal were collected from different regions of India and Egypt. The identity was confirmed and documented by Dr. P. Santhan, in-house taxonomist at Department of Pharmacognosy, R&D centre, Natural Remedies Pvt. Ltd., Bangalore, India. The freshly collected samples of leaves, aerial parts, and roots were washed, air dried, and stored at 4°C, protected from light and humidity before analysis.

2.2. Reagents and Chemicals. All HPLC grade solvents (acetonitrile, methanol, water, and *o*-phosphoric acid) were purchased from Rankem (Bangalore, India). Flavonoid glycosides (**1–3**) were isolated at Natural Remedies Pvt. Ltd. Identity and purity of the isolated constituents were confirmed by chromatographic (TLC, HPLC) and spectroscopic data (1D-NMR, 2D-NMR and HR-ESIMS) and by comparing with corresponding spectral data available in the literature [23, 24]. Purity of the isolated compounds was calculated to be 97.6%, 98.5%, and 99.5% for (**1–3**), respectively.

2.3. HPLC Instrumentation and Conditions. The HPLC system (Shimadzu, 2010CHT) consisted of a quaternary pump with vacuum degasser, thermostatted column compartment, autosampler, and UV detector. A reverse-phase column (Phenomenex Luna C_{18}, 5 μ, 250 X 4.6 mm) was used and the column temperature was maintained at 25°C. HPLC mobile phase, Solution A: potassium dihydrogen orthophosphate (0.136 g), was dissolved in 900 mL of HPLC grade water to which 0.5 mL of *o*-phosphoric acid was added and the volume was made up to 1000 mL with water. The solution was filtered through 0.45 μm membrane filter and degassed in a sonicator for 3 minutes; Solution B: acetonitrile. Mobile phase was run using gradient elution: 0 min, 10% B; in next 12 min to 20% B; in next 6 min to 45% B; in next 7 min to 80% B and maintained at 80% B for 3 min; in next 7 min to 45% B and further down to 10% B in next 5 min followed by an equilibration period of 5 min. The flow rate was 1.5 mL/min and the injection volume was 20 μL. The eluents were detected and analyzed at 350 nm.

2.4. Preparation of Standard Solutions. Stock solutions of marker constituents (**1–3**) were prepared at a concentration of 1.0 mg/mL in methanol. The calibration curves were prepared using solutions of different concentrations ranging from 10 to 550 μg/mL.

2.5. Sample Preparation. Dried and finely milled plant materials (1.0–2.0 g) of *W. somnifera* were extracted with the aid of reflux and sonication in 50 mL methanol. The supernatant was transferred to a flask. The procedure was repeated thrice and pooled extract was concentrated under vacuum and volume adjusted to 100.0 mL with methanol. Aliquots were filtered through 0.45 μm membrane filter before analysis.

2.6. Validation of the Method. The HPLC method was validated in terms of precision, accuracy, and linearity according to ICH guidelines [25] and the USP General Chapter ⟨1225⟩ *Validation of Compendial Procedures* [26]. The accuracy of the assay method was evaluated in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting serial dilutions of solutions of the

standards with known concentrations. The LOD and LOQ were calculated based on the signal-to-noise ratio of more than 3 times for LOD and 10 times for LOQ, respectively.

3. Results and Discussion

3.1. Optimization of the Chromatographic Conditions. The analytical method described in the USP monograph on ashwagandha is for the estimation of the withanolides content [27] and hence could not be utilized for detection of aerial parts in samples of powdered roots and extracts derived from roots, as the withanolides mentioned in the monograph are present in both aerial parts as well as in the roots of the plant. Therefore, a separate HPLC method was developed for estimation of (**1**–**3**), the "aerial parts specific" marker constituents, in different samples. During preliminary work, reverse-phase HPLC columns from different manufacturers were tested in order to optimize the conditions of separation. The best separation efficiency and peak shape were achieved on a reverse phase column (Phenomenex Luna C_{18}, 5 μ, 250 X 4.6 mm). Optimal chromatographic separation of (**1**–**3**) was achieved using gradient elution of a mobile phase consisting of acetonitrile and phosphate buffer. Acetonitrile was preferred over alcohol type of solvents because it enhanced the separation as well as offered relatively low back pressure. Selecting 350 nm as the detection wavelength, based on UV max of the isolated flavonoids, resulted in acceptable detector response for (**1**–**3**) with limited interference from other compounds present in the samples.

3.2. Method Validation. The HPLC method was validated for precision, accuracy, and linearity. The specificity was determined by injecting individual samples and ensuring no interference for analysis of (**1**–**3**) from other components of the sample matrices. Linear calibration plots were obtained over seven concentration levels (10–550 μg/mL). The results showed a linear correlation between the peak area and concentration (Table 2). The precision study used the plant sample PC/WS/02 and followed the validated procedure for sample preparation. The precision of the assay method was evaluated by carrying out three independent analyses on two different days. The % RSD of all three analytes was determined to be within the acceptable limit of 2.5%. Multiple injections illustrated that the results were reproducible and had a low standard deviation (Table 1). The RSD of assay results obtained in interday and intraday study was within 2.5% and showed a maximum of 1.38% for (**1**); 1.55% for (**2**) and 2.10% for (**3**) in intraday and 1.45% for (**1**); 1.58% for (**2**) and 1.22% for (**3**) in interday studies, confirming acceptable precision of the developed method. The accuracy of the method was determined by spiking samples with a known amount of all three standard compounds. The accuracy of the assay method was evaluated in triplicate at three concentration levels. The percentage recovery ranged from 90 to 108% for (**1**–**3**) (Table 2). The developed method exhibited acceptable performance in terms of sensitivity and baseline separation of all the marker compounds without interference from sample matrix.

Table 1: Results of system suitability tests[a].

Parameter	**1**	**2**	**3**
k'	2.830	3.047	3.517
A_S	1.217	1.383	1.035
N	9478	12265	8339
R_S	2.53	1.43	2.73
RRT	1.0	1.06	1.18
RSD (Rt)	0.11	0.14	0.16
RSD (AUC)	0.81	1.17	0.40

[a]Tests performed as per USP; Rt: Retention time; k': capacity factor; A_S: symmetry factor; N: number of theoretical plates; R_S: resolution; RSD (Rt): RSD of the retention time; RSD (AUC): RSD of the peak area.

Table 2: Results of validation of HPLC method.

Validation parameters	**1**	**2**	**3**
Specificity			
Peak purity (%)	>98	>98	>98
Linearity			
Conc. range, μg/mL	8.82 to 564.30	8.55 to 547.20	9.11 to 583.30
R^2	0.9996	0.9996	0.9995
LOD			
μg/mL	0.5	0.5	0.5
LOQ			
μg/mL	1.5	1.5	1.5
Precision			
Interday precision (RSD), %	1.45	1.58	1.22
Intraday precision (RSD), %	1.38	1.55	2.10
Accuracy: at three different concentrations in triplicate (n = 3)			
Recovery (%)	93–107	92–107	91–107

3.3. Analysis of "Aerial Parts Specific" Markers. Three samples of dried leaves designated as PC/WS/01, PC/WS/02, and PC/WS/03, three samples dried aerial parts designated as PC/WS/04, PC/WS/05, and PC/WS/06, and fifteen different samples of dried roots (collected from different sources in India) of *W. somnifera* were used. Additionally, dried samples of leaves and roots were obtained from Egypt. The samples were analyzed for the content of (**1**–**3**) by the developed HPLC method (Figure 2). The compounds were identified by spiking the samples with standard solutions of (**1**–**3**) and by comparison of UV spectra and retention times with those of the standards. All the fifteen samples of roots lacked (**1**–**3**), while all the tested samples of the leaves and aerial parts contained these flavonoid glycosides, indicating the latter's presence only in the tested leaves and aerial parts of *W. somnifera*. The concentrations of (**1**–**3**) in the samples of leaves and aerial parts are shown in Table 3. Ten different samples of commercially available standardized extracts of *W. somnifera*, labeled as "derived from roots", were tested

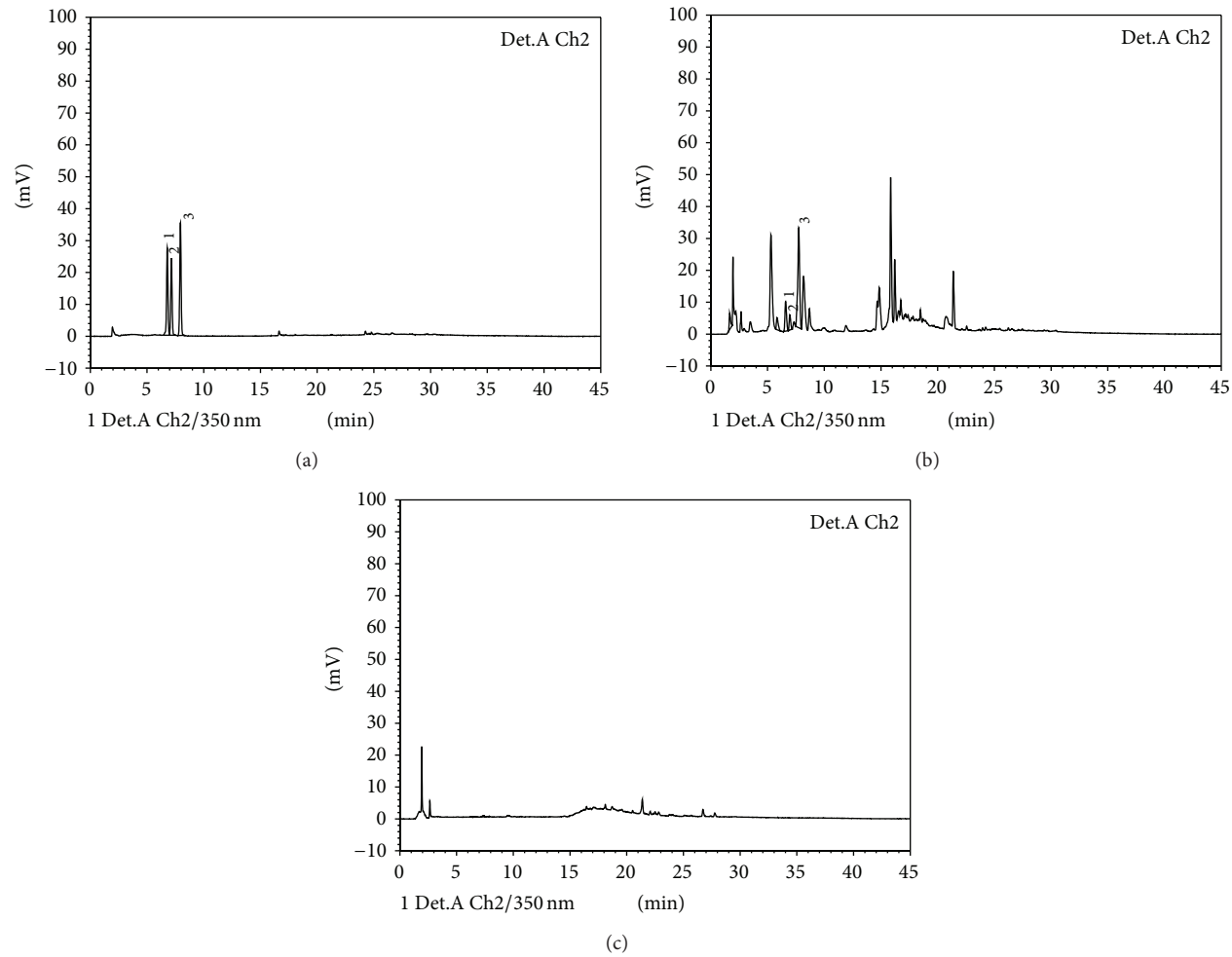


Figure 2: Representative chromatograms of (a) standard mixture of (**1**–**3**); (b) extract of *W. somnifera* leaves; (c) extract of *W. somnifera* roots. Chromatographic conditions: please see the experimental section.

Table 3: Detection of "aerial parts specific" compounds in different parts of *W. somnifera*.

Sample	Part used	Batch No.	"Aerial Parts Specific" markers		
			1	**2**	**3**
India	Leaves	PC/WS/01	0.046%	0.023%	0.069%
		PC/WS/02	0.032%	0.017%	0.049%
	Aerial parts	PC/WS/03	0.042%	0.020%	0.062%
		PC/WS/04	0.019%	0.008%	0.028%
		PC/WS/05	0.015%	0.007%	0.022%
		PC/WS/06	0.018%	0.007%	0.026%
	Roots	PC/WS-R/01-15[a]	Not detected	Not detected	Not detected
Egypt	Leaves	S10262	0.001%	0.154%	0.013%
		S10263	0.001%	0.233%	0.027%
	Roots	S10264	Not detected	Not detected	Not detected
		S10265	Not detected	Not detected	Not detected

[a]Fifteen collections of roots of *W. somnifera* from India were analyzed and were found devoid of (**1**–**3**).

Table 4: Detection of (**1**–**3**) in commercial samples of "standardized extracts" of roots of *W. somnifera*.

Description of samples	Identity of samples	"Aerial Parts Specific" markers		
		1	**2**	**3**
Extracts labeled as have been derived from roots	Sample 1	Not detected	Not detected	0.138%
	Sample 2	0.007%	0.006%	0.024%
	Sample 3	Not detected	Not detected	Not detected
	Sample 4	Not detected	Not detected	Not detected
	Sample 5	0.042%	0.037%	0.116%
	Sample 6	0.043%	0.035%	0.124%
	Sample 7	0.056%	0.049%	0.156%
	Sample 8	0.046%	0.042%	0.127%
	Sample 9	0.028%	0.022%	0.089%
	Sample 10	0.120%	0.089%	0.359%

for the presence (**1**–**3**). Interestingly, eight of the 10 extracts tested were found to contain the "aerial parts specific" marker constituents, pointing towards a possible use of aerial parts while preparing these extracts (Table 4). It may be noted that the aerial parts also contain the withanolides of roots specified in the USP monograph on ashwagandha and cost about 4 times less as compared to roots of *W. somnifera*.

4. Conclusions

An HPLC method was developed and validated for estimation of quercetin 3-*O*-robinobioside-7-*O*-glucoside (**1**), quercetin 3-*O*-rutinoside-7-*O*-glucoside (**2**), and kaempferol 3-*O*-robinobioside-7-*O*-glucoside (**3**) in the aerial parts of *W. somnifera*. Results from validation of the method showed satisfactory specificity, linearity, accuracy, precision, and reproducibility. The flavonoid glycosides (**1**–**3**) were absent in the samples of the roots analyzed. The method was found to be suitable for detection of aerial parts in extracts derived from roots of the plant. Analysis of various samples used in this study indicated the usefulness of the method in distinguishing between extracts of roots and aerial parts of *W. somnifera* and for routine detection of possible adulteration with aerial parts in the commercially manufactured extracts of roots of the plant.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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