

Article

Accumulation of Three Important Bioactive Compounds in Different Plant Parts of *Withania somnifera* and its Determination by the LC–ESI-MS-MS (MRM) Method

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Abstract

A comprehensive experiment was conducted to study the accumulation pattern and determination of three important bioactive compounds namely withaferin-A (WA), 12-deoxywithastramonolide (WO) and withanolide-A (WD) and its determination by the liquid chromatography/electrospray ionization tandem mass spectrometry (LC–ESI-MS-MS) method in root, stem, fruits and leaves of *Withania somnifera*. A rapid and sensitive LC–ESI-MS-MS method was developed and validated for the determination of these three important bioactive compounds, having same molecular weight. The multiple reaction monitoring method was established by two transitions for each analyte and intense transition used for quantification. Separation of the three analytes was achieved within a run time of 5 min on an RP-18 column using a mobile phase consisting of acetonitrile and 0.1% acetic acid in water in an isocratic condition. The developed method was validated as per the ICH guidelines. The developed method was found to be suitable for identification and quantification of WA, WO and WD in different plant parts such as roots, stems, fruits and leaves of *W. somnifera*. The accumulation of WA was highest in leaves samples (8.84 ± 0.37 mg/g) and it was 2.23, 5.85 and 27.26 times higher than its concentration in fruits, stems and roots, respectively. WO and WD contents were highest (0.44 ± 0.016 and 0.72 ± 0.016 mg/g, respectively) in root.

Introduction

Withania somnifera (L.) Dunal (Solanaceae), known as Aswagandha or Indian ginseng, is cultivated in India for centuries and used in more than 100 formulations. It has adaptogenic, antisedative, anticonvulsive and antioxidant activities. Excellent selective COX-2 inhibitory properties of this plant have also been reported (1–3). There are several bioactive constituents reported from different parts like root, leaves and fruits of *W. somnifera*. Four novel glycosides, a new withanolide and seven known withanolides were isolated from the methanol extracts of *W. somnifera* leaves (4). A dimeric withanolide, ashwagandhanolide, was also isolated from the roots of *W. somnifera*, which displayed growth inhibition against human gastric (AGS), breast (MCF-7), central nervous system (SF-268), colon (HCT-116) and lung (NCI H460) cancer cell lines (5). Two major withanamides,

i.e., A (WA) and C (WC) from the fruits of *W. somnifera*, were also reported for their ability to protect the PC-12 cells, rat neuronal cells, from β -amyloid-induced cell damage (6, 7).

Withanolides, a class of naturally occurring poly-oxygenated C₂₈ ergostane-based steroidal lactones, are reported to be the major phytochemicals attributing to the various bioactivities of this plant. Although, roots and leaves of this plant are mainly used for the pharmaceutical and nutraceutical preparations, withanolides have been reported from almost all parts of the plant.

The first withanolide, i.e., withaferin-A (WA), was isolated from *W. somnifera* (8). Recent review showed that about 360 numbers of naturally occurring withanolides have been isolated and identified from different plant species; many of them are the same names but different structures, whereas others have the same structures but different

names (9). However, very few works were reported on the quantification of compounds from this plant and systematic study of accumulation of important withanolides within this plant. An RP-HPLC method was developed for analysis of WA in the flowers, leaves and roots of *W. somnifera* (10). A HPTLC method for quantification of two withanolides from two morphotypes was also reported (11). There are HPLC and HPLC-UV-EIMS (SIM mode) methods for quantification of major withanolides (12, 13). However, these methods take more than 25 min per sample run. High-performance liquid chromatography–tandem mass spectrometry was developed for the determination of two biomolecules in mice plasma for application to pharmacokinetics after oral administration of *W. somnifera* aqueous extract (14). An LC–MS–MS method was developed for quantification of WA in plant extracts of *Withania* species by using a multiple reaction monitoring (MRM) mode, and the limit of detection (LOD) in the plants was 6 ng/g and the total analysis time was 10 min (15).

There is no comprehensive study for accumulation pattern of all the three bioactive molecules in leaves, fruits, stem and roots of *W. somnifera* by the LC–ESI–MS–MS (MRM) method. We conducted the experiment to study the accumulation of bioactive compounds and its simultaneous determination by the LC–ESI–MS–MS (MRM) method, which can rapidly identify and quantify three major bioactive compounds, i.e., WA, 12-deoxywithastramonolide (WO) and withanolide A (WD) in the leaves, fruits, stems and roots of *W. somnifera*. The chemical structures of WA, WO and WD are shown in Figure 1.

Experimental

Chemicals and materials

WA, WO and WD of purity 98.2, 96.5 and 96.7%, respectively, purchased from ChromaDex (CA, USA) were used as references. All solvents used were of HPLC grade (Merck, Mumbai, India). Ultra-pure distilled water with resistivity greater than 18 MΩ was used.

Withania somnifera plants were collected from the experimental field of the ICAR-Directorate of Medicinal and Aromatic Plants Research (ICAR-DMAPR), Boriavi, Anand, Gujarat (India), in the month of December. The plants were harvested at the stage of fruiting and it was partitioned into leaves, fruits, stems and roots. The plant materials were air-dried and powdered in an electric grinder. The powdered plant materials were used for the extract preparation.

Extraction of plant material

The methanol extracts of leaves, fruits, stems and roots were prepared as follows. One gram each of the powdered samples were extracted

with methanol (3 × 75 mL, 60 min), under reflux, on a temperature-controlled water bath maintained at 90°C. The extracts were combined, filtered and then evaporated under reduced pressure to get crude extracts from each plant part. The crude extracts from different plant parts of were thereafter dissolved in methanol and filtered through a 0.45-μm nylon membrane filter (Whatman, India). Stock solutions of the extracts were further diluted with methanol in order to obtain appropriate concentration.

Chromatographic conditions

A modular UFLC (Shimadzu Corporation, Kyoto, Japan), LC system was used, which consisted of two LC-20AD pumps, DGU-20A₃ degasser, SIL-20AC HT autosampler, a CTO-10ASvp column oven and CBM-20, a communications bus module. The analysis work was carried out by using mobile phase consisting of acetonitrile and 0.1% acetic acid in water (40 : 60) at a flow rate of 1.0 mL/min (split flow 80 : 20, 200 μL/min in an MS system) in an isocratic elution mode at 40°C column oven temperature. Chromatographic separation was achieved on a Chromolith RP-18 column (100 × 4.6 mm, Merck). Sample volume of 10 μL was injected into the LC–MS–MS system.

Mass spectrometric conditions

The API-2000 LC–MS–MS (Applied Biosystems/MDS SCIEX, Toronto, Canada) mass spectrometer was operated with ESI source coupled with an LC separation system. Analyst software (Version 1.4.2, Applied Biosystems/MDS SCIEX) was used for the control of equipment, acquisition and data analysis. MS parameters were optimized by constant infusing of a known concentration of standard WA, WO and WD. An MS scan was performed in both positive and negative modes and declustering potential was optimized. For the product ion spectrum (MS–MS) optimization, the optimized declustering potential was used with nitrogen as the collision gas to get prominent product ions.

Preparation of standards and working solutions

WA (1 mg/mL), WO (1 mg/mL) and WD (1 mg/mL) were prepared in HPLC grade methanol. Working stock solutions, 1,000 ng/mL of WA, WO and WD, were prepared by further dilution of the respective stock solutions in methanol.

Specificity, precision, accuracy and linearity

The specificity of the method was defined as non-interference in the regions of interests, for quantification of WA, WO and WD. Six replicates of blank samples were analyzed to ensure no interference with the mass transitions chosen for the three compounds. Method

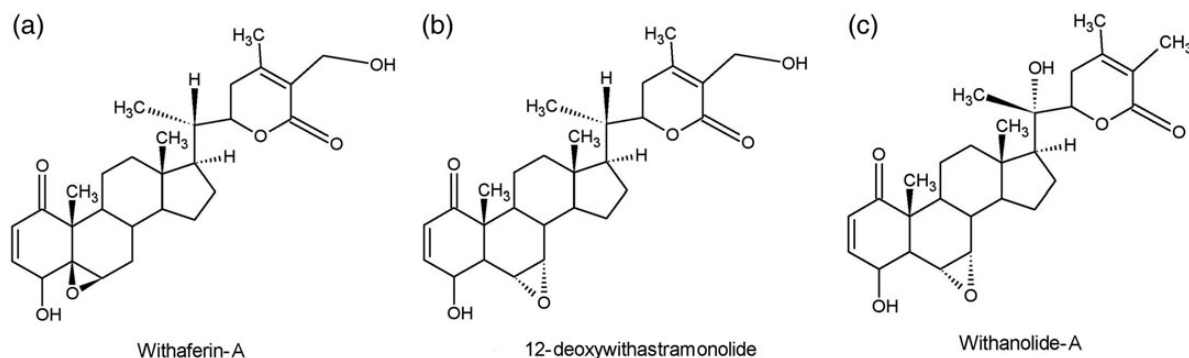


Figure 1. Structures of (a) withaferin-A, (b) 12-deoxywithastramonolide and (c) withanolide-A.

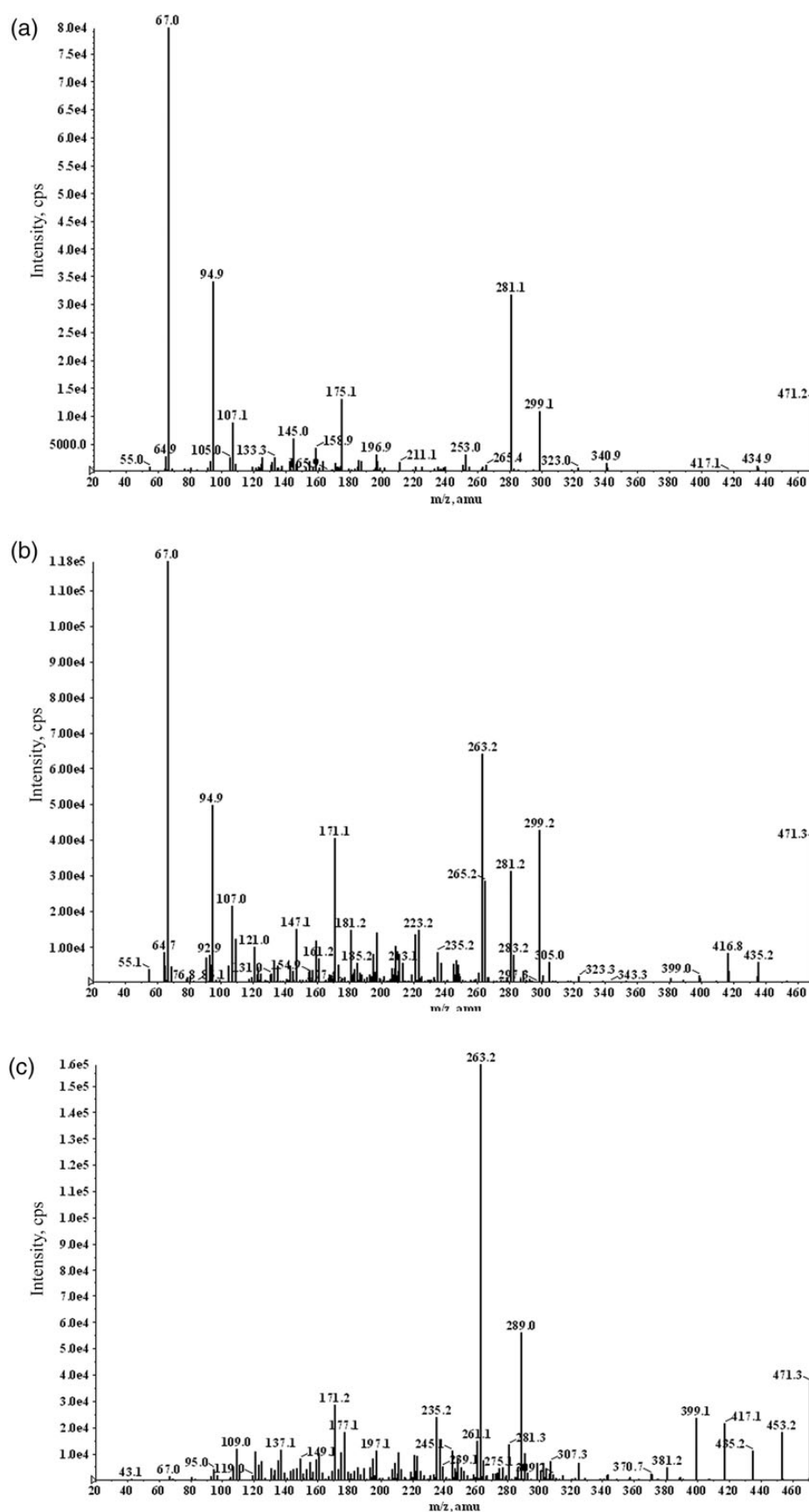


Figure 2. Product ion spectra of (a) WA, (b) WO and (c) WD.

reproducibility was evaluated by six injections of standards solutions and six replicate analyses of sample solutions, respectively. The precision of the method was expressed as relative standard deviation (RSD %) and it was calculated by six replicate injections of three different concentrations (low, medium and high) of standard WA, WO and WD. The accuracy of the method was tested by recovery studies by spiking three concentrations in root extracts sample.

The linear equation between the concentration of the standard injected and the peak area was expressed as $y = mx + c$, where y is the peak area; x is the concentration of the standard and m and c are constants.

LOD and LOQ

The LOD of the method for WA, WO and WD was the lowest quantity in the sample corresponding to three times the baseline noise (signal to noise, $S/N > 3$) and the limit of quantification (LOQ) was defined as the lowest concentration that can be determined with acceptable accuracy and precision at an S/N ratio of > 10 .

Results

Optimization of LC and MS conditions

LC conditions were optimized as described in the Experimental section using the mobile phase consisting of acetonitrile and 0.1% acetic acid in water (40 : 60) at a flow rate of 1.0 mL/min in an isocratic elution mode. Different columns, such as X-terra RP-18 (Waters), Symmetry RP-18 (Waters) and Chromolith RP-18 (Merck), were used; however, the best results were obtained on Chromolith RP-18 column (100 × 4.6 mm; 2 μm macro-, 13 nm micro-pore size). The mass spectrometric behaviors of the three compounds were investigated in both positive and negative ionization modes. In the negative ionization mode, no abundant deprotonated $[M-H]^-$ ion was observed for the three compounds. However, in the positive ionization mode, all the molecule showed abundant $[M+H]^+$; therefore, this mode was selected for further studies.

Optimization of MRM conditions

Standard solutions of WA, WO and WD were infused using a Hamilton syringe pump to the mass spectrometer to optimize the sensitivity for the $[M+H]^+$ ion, at m/z 471.2 for WA, 471.3 for WO and 471.3 for WD, respectively. The MS-MS spectra of the $[M+H]^+$ ion of WA showed major fragments at m/z 299.1, 281.1 and 175.1; WO showed fragments at m/z 299.2, 281.2, 265.1, 263.1 and 171.2 and for WD fragments at m/z 289.0 and 263.2 were observed. The product ion spectra of WA, WO and WD in the positive mode are given in Figure 2. The structure-fragmentation relationships in withanolides were reported as diagnostic product ions obtained from precursor $[M+H]^+$ by loss of water and cleavage/rearrangement of lactone moiety. Peak at m/z 281 was due to $[M+H-Lac-H_2O]^+$ and $[M+H-Lac-2H_2O]^+$ produced peak at 263 (16).

The ion spray voltage was set at 5,500 V and source temperature at 475°C. The curtain gas as a nitrogen gas, CAD, GS1 and GS2 (terms used in the Analyst software 1.4.2) were optimized and set at 10, 6, 60 and 50 psi, respectively. Analysis was carried out in the positive mode using MRM mode with two transition reactions used for each analyte, to validate the results. The transitions were (m/z 471.2 → 281.1; m/z 471.2 → 175.1) for WA, (m/z 471.3 → 263.1; m/z 471.3 → 265.1) for WO and (m/z 471.3 → 263.2; m/z 471.1 → 289.0) for WD. The intense daughter ion peak transitions were selected for quantification and the transitions m/z 471.2 → 281.1, 471.3 → 263.1 and m/z

Table I. Optimized MS-MS Parameters for WA, WO and WD of *W. somnifera*

Analytes	MRM transition (m/z)	Dwell time (ms)	DP (v)	CE (v)	EP (v)	CXP (v)
WA	471.2 → 281.1	200	70	37	10	7
	→ 175.1	200	70	25	10	6
WO	471.3 → 263.1	200	58	24	6	10
	→ 265.1	200	70	20	10	12
WD	471.3 → 263.2	200	70	20	10	12
	→ 289.0	200	70	18	10	10

DP, declustering potential; CE, collision energy; EP, entrance potential; CXP, collision cell exit potential; v, volt.

471.2 → 263.2 were used for quantification of WA, WO and WD, respectively. The optimizations of MS-MS fragmentation condition were studied. Typical MS conditions of each component are presented in Table I.

Method validation

WA, WO and WD have same molecular weight (470 Da); however, they have different retention times 3.31 min for WA, 3.82 min for WO and 4.40 min for WD. The specificity of the developed method was expressed by MRM chromatograms of WA, WO and WD obtained from the reference standards (Figure 3) as well as from extract samples of *W. somnifera* and blank.

The reproducibility of the retention times of WA, WO and WD under optimized LC-MS-MS conditions was investigated by repeated injections ($n = 10$) of a mixture of standards at a concentration of ng/mL. The RSD % of retention times (min) for WA, WO and WD were 0.15, 0.33 and 0.43%, respectively (Table II). All the three analytes in the present study eluted within a run time of 5.0 min with good resolution, thereby demonstrating that the method is rapid for the analysis of the withanolides without using any buffer in comparison with the earlier reports.

Based on an S/N ratio of 3, the LODs in the MRM mode were 3.33 ng/mL for WA and WO and 6.67 ng/mL for WD, and it was found that the method is more sensitive that can detect analytes up to part per billion (ppb) level. A good linearity of the method was found over the investigated calibration range of 10–500, 10–120 and 20–120 ng/mL for WA, WO and WD, respectively, based on six level calibration curves. The independent injections were carried out for every calibration points. Typically, the coefficients of correlations (r^2) of this method were above 0.99 for WA, WO and WD (Table II).

The precision of the method was expressed as RSD (%) and was calculated by six replicate injections of the three different concentrations (low, medium and high) of standard, i.e., WA (100, 300 and 500 ng/mL), WO (20, 60 and 100 ng/mL) and WD (40, 80 and 120 ng/mL). The inter- and intraday RSD (%) of the developed LC-MS-MS method were in the range of 2.37–4.84, 1.58–5.96 and 1.17–3.73 for WA, WO and WD, respectively (Table III). The accuracy of the method was defined in terms of recovery of analytes and the mean recovery percentage of WA, WO and WD was found to be 102.57, 102.78 and 99.73%, respectively, at three different concentration levels (Table III).

Quantitative determination and accumulation pattern of WA, WO and WD in different plant parts of *W. somnifera*

Under the optimum LC-MS-MS conditions, the presence of three analytes WA, WO and WD in the root, stem, fruit and leaves extracts of

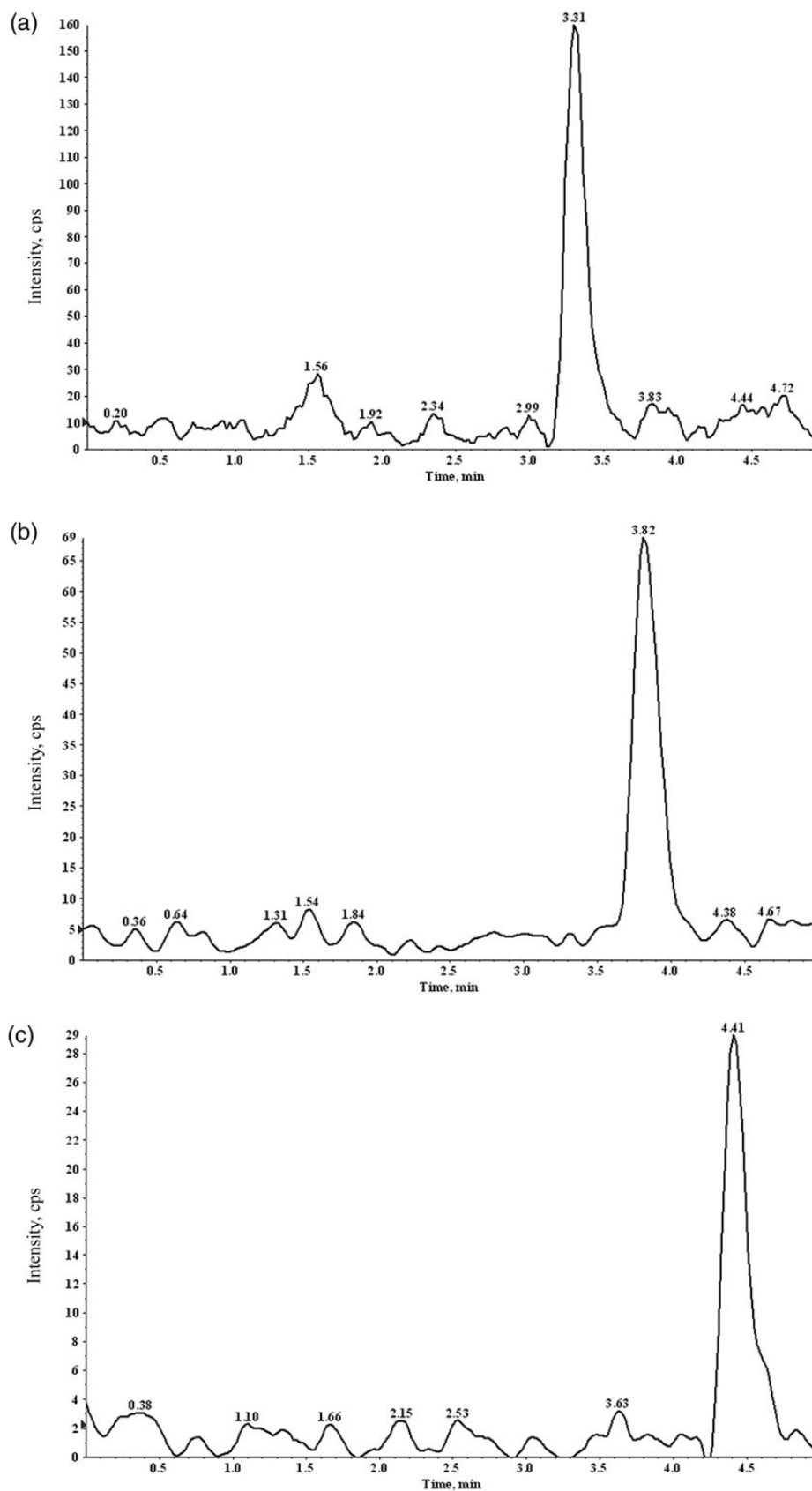


Figure 3. Representative MRM chromatograms of (a) WA, (b) WO and (c) WD.

Table II. Response Characteristics of WA, WO and WD Using LC–MS–MS

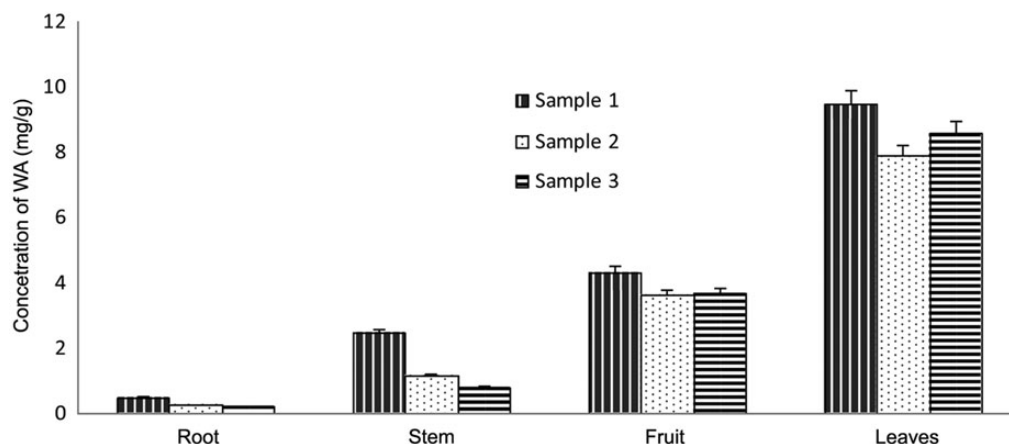
Analytes	Retention time (min)		Calibration equation ^a	R ²	Linearity range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
	Mean	RSD (%)					
WA	3.31	0.15	$Y = 48.4x + 498$	0.9906	10–500	3.33	10
WO	3.82	0.33	$Y = 65.7x + 355$	0.9901	10–120	3.33	10
WD	4.40	0.43	$Y = 8.66x - 17.2$	0.9869	20–120	6.67	20

^aIn the calibration equation, x represents concentration of the analytes (ng/mL) and y represents the peak area (count per second).

Table III. Precision (RSD %) and Accuracy (Recovery %) of the Developed Method at Three Different Concentrations

Analytes	Conc. (ng/mL)	Intraday (RSD %)	Interday (RSD %)	Added conc. (ng/mL)	Measured conc. (ng/mL)	Recovery (%)	Mean recovery (%)
WA	100	4.78	4.84	100	103.93	103.93	102.57
	300	2.82	4.16	200	203.71	101.86	
	500	3.75	2.37	400	407.71	101.93	
WO	20	3.94	5.96	20	20.68	103.42	102.78
	60	1.86	4.25	60	62.74	104.57	
	120	1.58	3.38	100	100.36	100.36	
WD	40	3.73	3.55	40	38.96	97.42	99.73
	80	3.04	3.34	80	62.77	104.62	
	120	1.17	1.20	100	97.17	97.17	

Conc., concentration.

**Figure 4.** Accumulation of WA (on dry weight basis) in different plant parts of *W. somnifera*. Each bar represents the mean \pm standard deviation.

W. somnifera was successfully identified and quantified based on ion transition used for MRM detection as well as by their retention times.

The validated method was applied for analysis of WA, WO and WD in extracts of roots, stems, fruits and leaves of *W. somnifera*. The results showed that the amount of all the three bioactive compounds significantly varied in different plant parts of *W. somnifera*.

WA content ranged from 0.317 to 8.64 mg/g of dry weight of plant samples, i.e., roots, stems, fruits and leaves. The accumulation of WA was recorded highest in leaves, i.e., 8.84 ± 0.37 mg/g (average values all the samples), and it was 2.23, 5.85 and 27.26 times higher than that of fruits, stems and roots, respectively (Figure 4). WO concentration was highest in root samples (0.44 ± 0.016 mg/g) and it was 4.49, 10.96 and 25.28 times higher than that in fruits, stems and leaves,

respectively (Figure 5). Similarly, WD concentration was maximum in root, i.e., 0.72 ± 0.016 mg/g (Figure 6) and it was 17.64 and 27.96 times higher than that in fruits and stems, respectively. Khajuria *et al.* (13) also reported that percentage of WD was maximum in root. WD was not detected in leaves. Accumulation of phytochemical in growth stages vary within organs and with development stage, as the synthesis and accumulation of bioactive principles are regulated by genetic and environmental factors. Studies conducted by Saravanan *et al.* (17, 18) in plants grown at different light intensity and plant population density showed variation in andrographolide content in *Andrographis paniculata* plant parts. These studies also support our results. This information will help for selection of plant parts according to requirement of bioactive compounds.

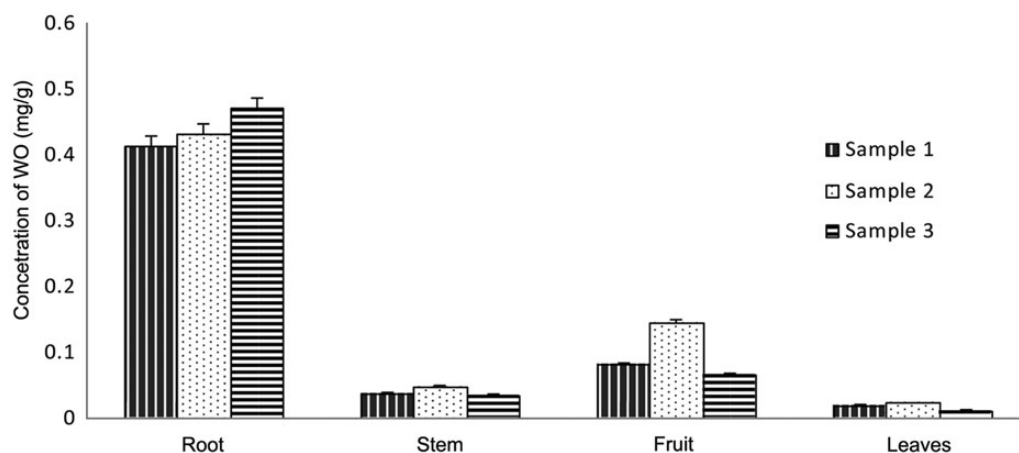


Figure 5. Accumulation of WO (on dry weight basis) in different plant parts of *W. somnifera*. Each bar represents the mean \pm standard deviation.

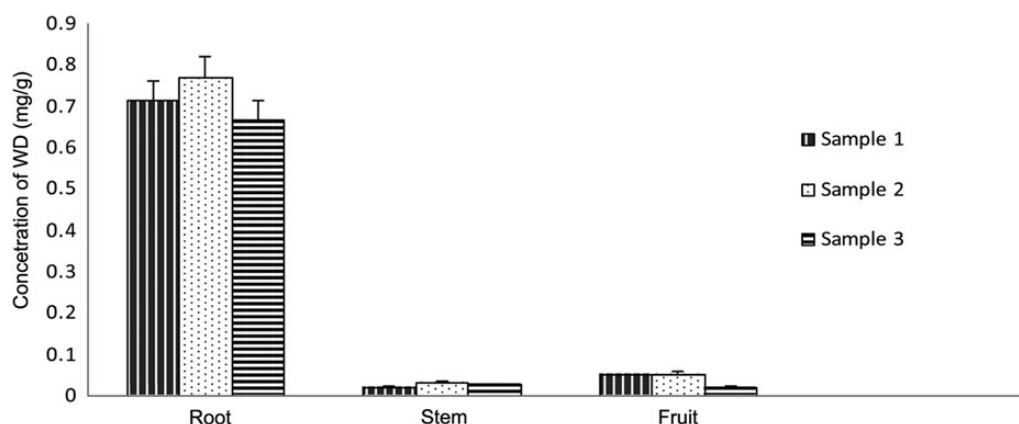


Figure 6. Accumulation of WD (on dry weight basis) in different plant parts of *W. somnifera*. Each bar represents the MEAN \pm standard deviation.

Conclusion

LC-ESI-MS-MS (MRM) method was developed and validated for identification and quantification of three major bioactive compounds, i.e., WA, WO and WD in *W. somnifera*. The developed and validated method is rapid, precise, specific and more accurate. This method can be used for screening of these three bioactive compounds from plant samples and formulations. This study confirmed that the accumulation of withanolides varied within a plant. WA was recorded highest in leaves in comparison to other plant parts. WO and WD were higher in the root part of the plant.

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