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Phytochemical and genetic analysis in selected chemotypes of *Withania somnifera*

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Abstract

The main active components and genetic profile of 15 selected accessions of *Withania somnifera* Dunal. were analysed. Ethanolic extract of the dried roots/leaves of the plant was concentrated under pressure at 50 ± 5 °C and was analysed for main compounds (withanolides and withaferin A) by HPLC. All the main components were found to be present in accessions (AGB 002, AGB 009, RSS 009, RSS 033). Correlation of these main components with their genetic factors, was undertaken using AFLP (amplified fragment length polymorphism) markers. Among 64 primers 7 yielded optimum polymorphism. A total of 913 polymorphic peaks were generated using these primers. Jaccard's similarity coefficient indicated that accessions having almost the same active compounds clustered together. The present study demonstrates that AFLP can be successfully used to resolve the correlation of AFLP data with the presence of secondary metabolites.

Keywords: *Withania somnifera*; AFLP; TLC; HPLC; IP; NMR; MS

1. Introduction

Withania somnifera (Ashwagandha) is one of the most valued plants in Ayurveda and is commonly used in Indian traditional health care systems. The plant is said to have a potential property of pacifying 'Vata' in herbal drugs (Anonymous, 1998; Singh et al., 2001; Dhuley, 2001). Singh et al. (2001) compared therapeutic value of its roots with *Panax ginseng* and found them similar in many respects. A large number of withanolides have been identified in *Withania* roots and leaves (Glotter et al., 1973; Eastwood et al., 1980; Nittala and Lavie, 1981). Some of these, like withaferin A have been associated with anti-inflammatory (Sethi et al., 1970) and immunosuppressive (Shohat et al., 1978) properties, whereas sitoindosides IX and X are immunostimulatory (Ghosal et al., 1989). Withanolide D has antitumour activity (Leyon and Kuttan, 2004) and sitoindosides VII and VIII are antioxidants (Bhattacharya et al., 1997; Panda and Kar, 1997). Other withanolides, including their glycosylated products are reported to have immunomodulatory and other activities (Zhao et al., 2002). Since different therapeutic activities are associated with different constituents, it is important to determine and compare secondary metabolite contents and genetic profiles of the selected 15 accessions to develop an efficient molecular tool for designing germplasm for targeted therapeutic value.

Chemotypes, with several-fold variability in the content of individual withanolides have been reported in *W. somnifera* (Singh and Kumar, 1998). However, little attempt has been made to identify the biochemical pathway for chemical transformation of the steroid lactones in

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W. somnifera. Literature scan suggests that there are various new withanolide glycosides (withanosides) isolated from Indian ashwagandha roots (Matsuda et al., 2001). Several mono- and poly-herbal products of *W. somnifera* which are commercially available in the Indian market were quantitatively analysed by Sangwan et al. (2004) for a number of chemical constituents and found a wide variation in seven of them.

Molecular techniques play an increasingly important role in the management and utilization of plant genetic resources. The development of isozymes and DNA databases is a prerequisite for the protection and documentation of any variety/species. There are many molecular markers which can be linked to genes of interest, allowing indirect selection of the desired genotype and also for the identification of commercial varieties. Identification of DNA markers that can correlate DNA fingerprinting data with quantity of selected phytochemical markers associated with that particular plant, would have extensive applications in quality control of raw materials. Among various molecular techniques, AFLP has been proved to be an efficient tool in the identification of diagnostic or specific markers.(Vos et al., 1995; Campbell et al., 2003; Diaz et al., 2003). Negi et al. (2000) investigated the genetic variation in inter- and intraspecific population of *W. somnifera* using AFLP markers. AFLP is characterized by high reproducibility and high multiplex ratio and does not require prior sequence information.

In the present study, an attempt was made to correlate the genetic data obtained from AFLP of 15 selected accessions with different components from the leaves of germplasm. Such studies would establish the power of DNA fingerprinting in identifying the genes associated with secondary metabolism in medicinally important plants for breeding programmes, for pharmacological studies and eventually for intellectual property rights.

2. Results and discussion

2.1. Phytochemical Analysis

The 15 accessions of W*ithania somnifera* selected in this study differed widely both qualitatively and quantitatively with respect to withanolides/glucowithanolides (Tables 1 and 2) respectively, whose structures are depicted in Fig. 1. Withaferin-A was found to be present in the leaves as well as in the roots in all the accessions of *W. somnifera* belonging to both RRL and CIMAP. But withanone was exceptionally absent in both leaves and roots of AGB 015 and AGB 025 but for RSS 030, RSS 027 and RSS 018, it is found to be present in roots while absent in leaves. The content of withanolide was found to be more in roots than in leaves (RSS 001, RSS 006, RSS 008, RSS 018, RSS 028, RSS 030, RSS 033, AGB 015 and AGB 030), whereas the content of other components are averaged low in roots as compared to leaves in all the accessions. Among RRL and CIMAP accessions, AGB 009 and RSS 08 was found to have all the seven constituents in its roots and leaves. The presence of seven isolated withanolides (Fig. 2) fluctuated both in leaves as well as in roots and is possibly due to varying degree of synthesis and utilization of these compounds in the plant. However, the product of commerce is the root, which contains the active compounds of therapeutic value and mostly different withanolides. A representative HPLC profile showing all the seven withanolides are presented for AGB 002 L (leaves) and R (root) (Fig. 2 b + c and Table 2) and was compared with HPLC graph for markers used for chemoprofiling (Fig. 2a).

Table 1
Presence and absence of major constituents in (leaves/roots) of selected 15 accessions of *Withania somnifera*

Accessions	WS-1[a]	WS-2	WS-3	WSC	WSCO	WSG-3	WSGP
AGB 002 (L)	–	+	+	+	+	+	+
AGB 002 (R)	+	+	+	+	–	+	–
AGB 009 (L)	+	+	+	+	+	+	+
AGB 009 (R)	+	+	+	+	+	+	+
AGB 015 (L)	+	–	+	–	+	+	+
AGB 015 (R)	+	–	+	+	+	+	+
AGB 025 (L)	–	–	+	+	–	+	–
AGB 025 (R)	+	–	+	–	–	+	+
AGB 030 (L)	+	+	+	+	+	+	+
AGB 030 (R)	+	+	+	+	–	–	+
RSS 001 (L)	+	+	+	+	+	+	+
RSS 001 (R)	+	+	+	+	+	+	+
RSS 004 (L)	–	+	+	–	+	+	+
RSS 004 (R)	+	+	+	–	+	+	+
RSS 008 (L)	+	+	+	+	+	+	+
RSS 008 (L)	+	+	+	+	+	+	+
RSS 009 (L)	+	+	+	+	+	+	+
RSS 009 (R)	+	+	+	+	+	+	+
RSS 016 (L)	–	+	+	+	+	+	+
RSS 016 (R)	+	+	+	+	+	+	+
RSS 018 (L)	+	–	+	+	+	+	+
RSS 018 (R)	+	+	+	+	+	+	+
RSS 027 (L)	–	–	+	–	+	+	+
RSS 027 (R)	+	+	+	+	+	+	+
RSS 030 (L)	–	–	+	–	+	+	+
RSS 030 (R)	+	+	+	+	+	+	+
RSS 033 (L)	+	+	+	+	+	+	+
RSS 033 (R)	+	+	+	+	+	+	+
RSS 041 (L)	–	+	+	+	+	+	+
RSS 041 (R)	+	+	–	+	+	–	+

[a] WS-1, Withanolide-A; WS-2, withanone; WS-3, withaferine-A; WSC, withastramonolide, SCO, 27-hydroxywithanone; WSCG-3, withanoside; WSG-P, physagulin.

2.2. AFLP analysis

Out of 64 AFLP primer combinations, 7 primers were found to be optimum for the uniform distribution of peaks in the region analyzed from 70 to 350 bases enabling them to be used as polymorphic markers. A total of 1050 peaks were generated using the selected 7 primer combinations. An average of 79.7% polymorphic peaks were detected among all the 15 accessions. Only consistent and reliable fragments were scored. Maximum number of peaks (212) were found with E_{ACA}–M_{CAA} primer combination (Table 3). The

Table 2
Concentration of main chemical constituents (g/100 g d.w.b) in various accessions of *Withania somnifera*

Sample	WS-1	WS-2	WS-3	WSC	WSCO	WSG-3	WSG-P
AGB002L	–	0.043	0.164	0.026	0.046	0.016	0.003
AGB002R	0.013	0.013	0.008	0.007	–	0.008	–
AGB009L	0.025	0.126	0.217	0.013	0.028	0.050	0.025
AGB009R	0.117	0.005	0.007	0.009	0.001	0.010	0.012
AGB015L	0.005	–	0.567	–	0.020	0.059	0.021
AGB015R	0.015	–	0.044	0.004	0.001	0.042	0.017
AGB025L	–	–	0.174	0.002	–	0.012	–
AGB025R	0.019	–	0.007	–	–	0.003	0.017
AGB030L	0.009	0.127	0.248	0.125	0.147	0.084	0.007
AGB030R	0.029	0.011	0.002	0.002	–	–	0.006
RSS-01L	0.006	0.192	0.356	0.073	0.047	0.011	0.046
RSS-01R	0.017	0.003	–	0.001	0.001	0.001	0.013
RSS-04L	–	0.001	0.400	–	0.002	0.005	0.020
RSS-04R	0.012	0.004	0.016	–	0.004	0.001	0.01
RSS-08L	0.005	0.293	0.029	0.001	0.063	0.001	0.003
RSS-08R	0.024	0.002	0.001	0.001	0.001	0.003	0.002
RSS-09L	0.017	0.320	0.026	–	0.047	0.001	0.002
RSS-09R	0.009	–	–	0.008	–	0.002	0.006
RSS-16L	0.011	0.277	0.256	0.052	0.042	0.006	0.086
RSS-16R	–	–	–	0.008	–	0.007	0.010
RSS-18L	0.002	–	0.403	0.123	0.038	0.024	0.019
RSS-18R	0.043	0.035	–	0.003	0.003	0.003	0.002
RSS-27L	–	–	0.288	–	–	0.011	0.076
RSS-27R	0.034	0.001	–	0.001	–	0.004	0.001
RSS-30L	–	–	0.240	–	0.002	0.003	0.012
RSS-30R	0.026	0.005	0.003	0.001	0.003	0.002	0.009
RSS-33L	0.006	0.122	0.088	0.008	0.035	0.004	0.013
RSS-33R	0.020	0.003	0.001	–	0.001	0.006	0.001
RSS-41L	0.002	–	0.348	0.038	0.017	0.055	0.055
RSS-41R	0.011	–	–	0.004	0.001	0.017	0.003

maximum polymorphic peaks (184) were observed with E_{ACA}–M_{CTT} primer combination, whereas, the maximum polymorphic percentage was observed with E_{ACA}–M_{CTG} (90.79%) which is similar to E_{ACA}–M_{CTT} (89.75%). Average number of peaks with each primer was 163.57 and average polymorphic peaks were found to be 130.42. One peak of 152 size was found to have some correlation with withanone/withaferin as this peak was commonly present when both the components were found in an accessions, thus it may be predicted that this may represent a gene that influences the pathway leading to biosynthesis of withanolides. Smelcerovic et al. (2006) have demonstrated the correlation for secondary metabolite content with RAPD data among six *Hypericum* species from Serbia. However, AFLP gives wider access to the genome with high multiplex ratio and both theoretically and empirically is much superior to RAPD in information content and reliability.

The AFLP data were used to create a phenetic dendrogram (Fig. 3) using NTSYS-PC statistical package (v. 2.1, Rohlf, 1992). At 75% similarity level almost three quarters of the accessions have grouped together in cluster I according to their chemical profile as represented by E_{ACA}–M_{CTG} primer combination (Table 3), whereas, RSS 018 and RSS 030 have individually branched out in II and III, respectively. Cluster I which comprising the rest of the accessions was found to be divided into subclusters due to the presence of other compounds with few exceptions which are absent or present in one or the other (Table 2). RSS 033 and AGB 030 are closely allied as their similarity coefficient is $r = 0.83$ and both have withanolide and withaferin A as main active compounds. The parallelism between the distribution of withanolides and the phenetic dendrogram generated using AFLP markers suggests an appreciable degree of congruence between the two. Among the AFLP peaks which were presumed for withaferin A and withanone, only one peak of size 152 (amplified by E_{AGG}–M_{CTT} primer combination) was found to be common and may represent the gene that influences the pathway leading to the biosynthesis of both the molecules. The other peaks selected for withaferin A were found to be different from withanone.

The present study has implications for AFLP as a useful molecular tool for molecular marker testing in different accessions of *W. somnifera* with a high-resolution and reproducibility, as it can also resolve the correlation of AFLP data with the amount of secondary metabolites. Though our study suggests that sufficiently large data on AFLP fragments needs to be developed on a number of chemotypes that express a wide array of metabolites. Such data could be valuable in selecting elite accessions for designing plants.

3. Experimental

3.1. Materials

Fifteen accessions of *W. somnifera* maintained in germplasm repository at Regional Research Laboratory, Jammu and Central Institute for Medicinal and Aromatic Plants, Lucknow were used in the present study. These accessions were selected out of a large collection of more than 100 accessions from different geographical locations in India. Individual accessions were multiplied for two generations by bulking the seeds from single plants grown in isolated plots. The germplasm comprised phenotypically homogeneous populations. Out of these, 15 accessions were selected (Five from RRL coded as AGB 002, AGB 009, AGB 015, AGB 025, AGB 030 and 10 from CIMAP coded as RSS 001, RSS 004, RSS 008, RSS 009, RSS 016, RSS 018, RSS 027, RSS 030, RSS 033, RSS 041) on the basis of widely contrasting levels of different chemical compounds namely, withaferin A, withanone and withanolides, which are the main active compounds. The fresh leaves were collected from all plants at full bloom stage and were used for DNA fingerprinting whereas, both roots and leaves were dried at room temperature for their phytochemical analysis.

3.2. Isolation of pure withanolides/glucowithanolides which were used as markers for chemoprofiling of the extracts by HPLC

Dried and powdered roots/leaves of *W. somnifera* (AGB 002) were percolated four times with ethanol:water (1:3) at

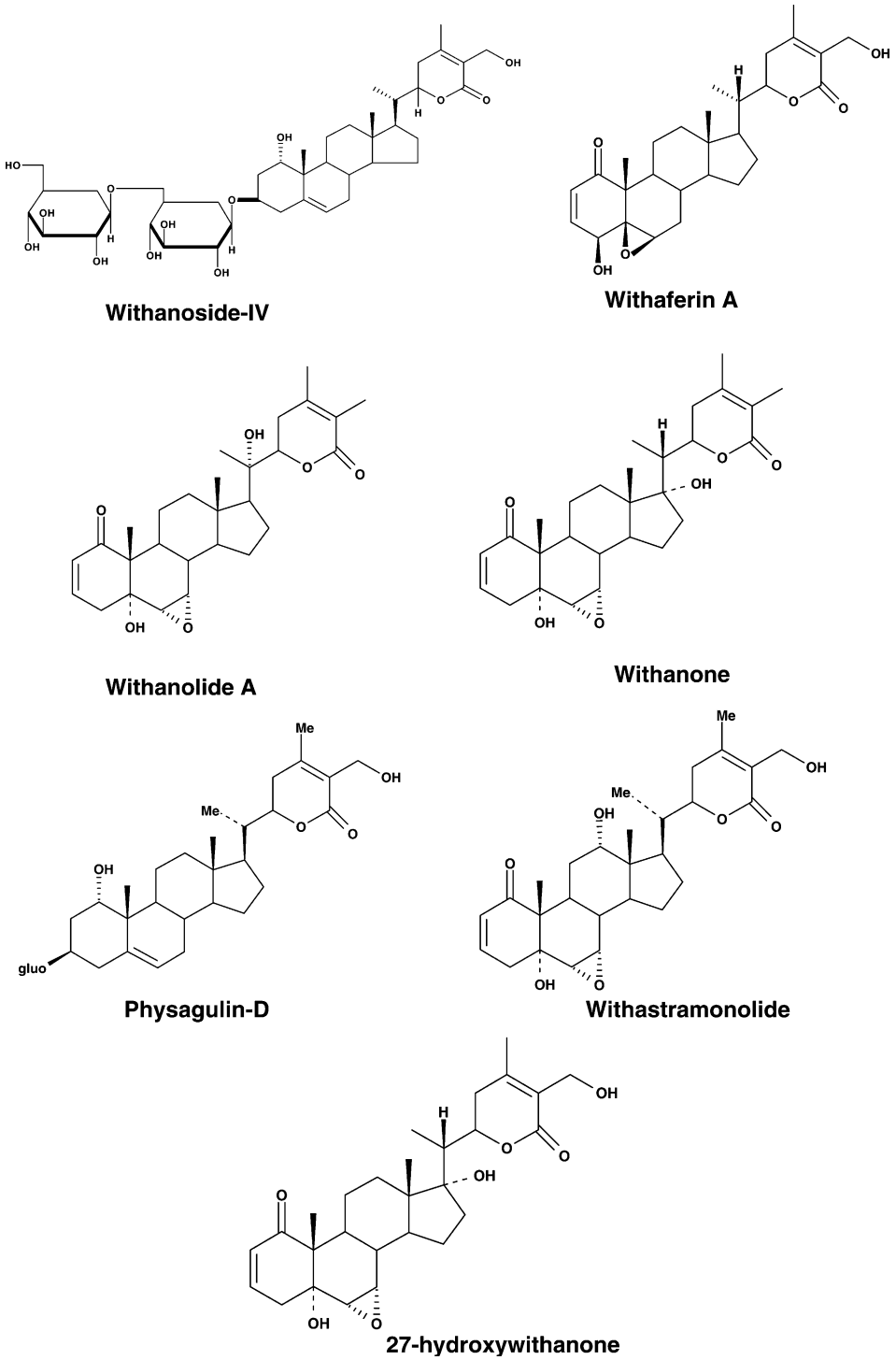
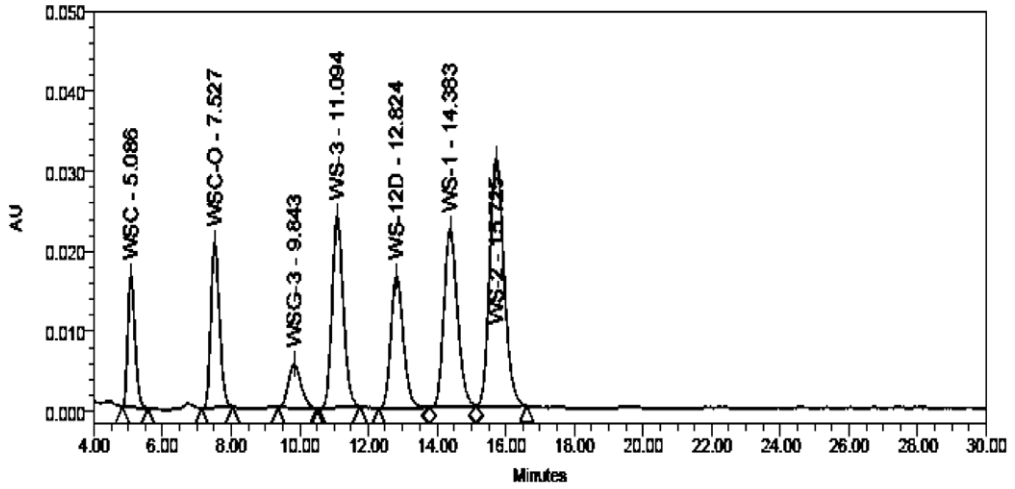


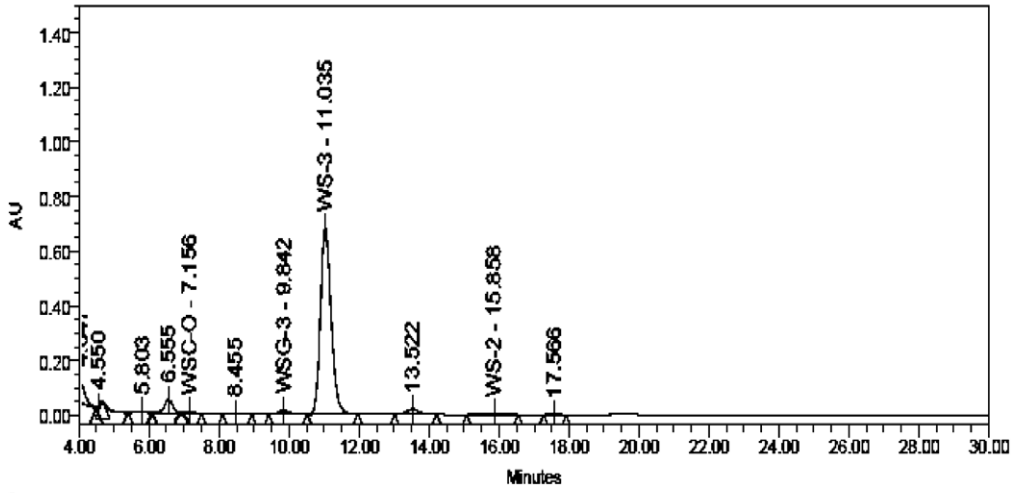
Fig. 1. Structures of the main active compounds isolated from the roots and leaves of *Withania somnifera*.

room temperature. The combined extracts were filtered, centrifuged and concentrated to 1/6th of the original volume under reduced pressure at a temperature of $50 \pm 5°$. Finally, the extract was completely dried under vacuum in desiccator. Total yield of the extract is 15–17%.

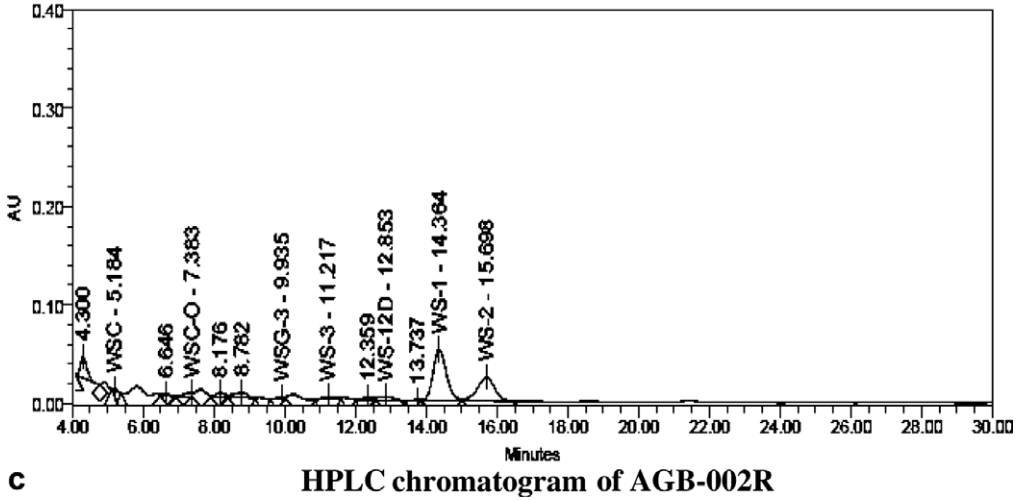
For the isolation of withanolides/glucowithanolides, the aqueous ethanolic extract (1:3) was dissolved in water and the solution was successively extracted with chloroform and *n*-butanol in a separating funnel. Both chloroform and *n*-butanol fractions were separately concentrated under reduced pressure to yield the residues containing withanolides and glucowithanolides. The withanolides were monitored by TLC system $CHCl_3$:MeOH (19:1) and glucowithanolides by TLC system $CHCl_3$:MeOH (4:1).



a HPLC chromatogram of seven markers



b HPLC chromatogram of AGB-002L



c HPLC chromatogram of AGB-002R

Fig. 2. HPLC profile of different withanolide markers in *Withania somnifera*: (a) standard markers; (b) AGB 030 leaf extract; (c) AGB 030 root extract.

3.3. Determination of secondary metabolic contents

The isolation and purification of withanolides/glucowithanolides (WS-1, WS-2, WS-3, WSC, WSCO, WSG-3 and WSG-P) obtained from the chloroform and *n*-butanol extracts were carried out by a combination of different separation techniques such as column chromatography, preparative TLC and centrifugal adsorption chromatography

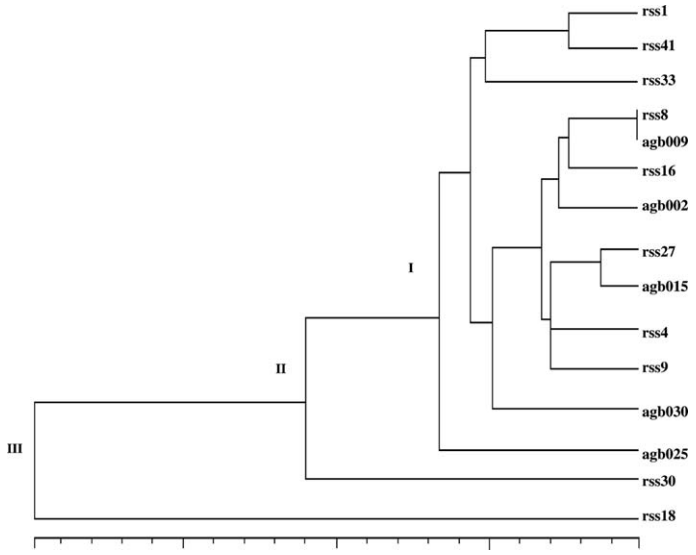


Fig. 3. Phenetic dendrogram based on AFLP data using UPGMA cluster analysis of 15 accessions of *Withania somnifera*.

Table 3
Primer combinations employed to detect AFLP markers among 15 selected accessions of *Withania somnifera*

Primer combination	No. of peaks	Polymorphic peaks	Percent polymorphism
E_{AAC}–M_{CTC}	128	103	80.46
E_{ACT}–M_{CTT}	205	184	89.75
E_{AGG}–M_{CAA}	203	136	67.00
E_{AGG}–M_{CTT}	162	121	74.69
E_{ACA}–M_{CAA}	212	165	77.83
E_{ACA}–M_{CTG}	163	148	90.79
E_{ACG}–M_{CTG}	72	56	77.77
Total	1050	913	79.70

(chromatotron) followed by crystallisation in appropriate solvents. The isolated pure withanolides/glucowithanolides were identified with the help of IR, NMR and MS data (Ray and Gupta, 1994; Singh and Kumar, 1998).

The bioactive extracts were standardised on the basis of five withanolides and two glucowithanolides using HPLC protocol (Fig. 2) and the results are mentioned in Table 2.

3.4. Sample preparation

An accurately weighed quantity of the dried aqueous alcoholic extracts was dissolved in known volume of HPLC grade methanol:water (1:1). The samples were filtered through millipore micro-filter (0.45 μm) and then injected into the HPLC system. Stock solutions of the pure reference compounds were prepared in HPLC grade methanol and stored in refrigerator in dark at 4 °C. From the stock solutions working solutions in the concentration range of 17.5 μg/ml to 35.0 μg/ml for each reference compound were prepared by dilution with HPLC grade methanol. These working solutions of all the reference compounds were mixed together in equal volumes for further analysis.

3.5. Quantification

The compounds exhibited linear response in the concentration range of 17.5 μg/ml to 35.0 μg/ml and the calibration curves were prepared by using multipoint calibration curve method. Working solutions after mixing were injected in different concentrations. Excellent calibration curves were obtained for WSC($r^2 = 0.998886$), WS-3($r^2 = 0.995346$), WS-1($r^2 = 0.995150$), WS-2($r^2 = 0.999155$), WSCO($r^2 = 0.995145$) and two glucowithanolides viz., WSG-3 ($r^2 = 0.999588$), and WSG-P($r^2 = 0.996404$). Calibration curves were determined on the basis of six levels of concentration of each standard in the mixture.

3.6. DNA extraction

Freshly harvested leaves were used to extract total genomic DNA from individual plants by standard CTAB method (Doyle and Doyle, 1990). The leaves were washed in running tap water and then dried in between filter paper before using for DNA isolation. Estimation of DNA quantitatively and qualitatively was done by spectrophotometer (Bio Photometer, Eppendorf, Germany) and agarose gel (0.5%) electrophoresis, respectively.

3.7. AFLP analysis

AFLP procedure was performed according to the method described by Vos et al. (1995). The AFLP plant mapping kit by Applied Biosystems, USA was employed for the experiment. Genomic DNA was digested *Eco*RI and *Mse*I and the DNA template was prepared for subsequent PCR amplification by ligating it with double stranded adaptors. These adaptors served as primer binding sites for low level selection in preselective amplification of the restriction fragments. To carry out the AFLP reaction, an

enzyme master mix was prepared containing 10 μl of 10× T_4 DNA ligase buffer along with ATP, 10 μl of 0.5 M NaCl, 5 μl of (10 mg/ml) BSA, 5 μl of (20 U/μl) of *Mse*I, 25 μl of (200 U/ul) of *Eco*RI, 40 μl of (3 U/μl) of T_4 DNA ligase for 10 reactions. Total volume was made upto 100 μl with distilled water. This mixture was called EMM (Enzyme master mix). The restriction ligation mixture consisted of 1.0 μl of T_4 DNA ligase buffer with ATP, 1.0 μl of 0.5 MNaCl, 0.5 μl (10 mg/ml) BSA, 1.0 μl of *Mse*I adaptor, 1.0 μl of *Eco*RI adaptor (Applied Biosystems), 1.0 μl of EMM and about 0.5 μg of DNA sample. The reaction was incubated overnight at 37 °C which was subsequently diluted 20-fold with TE buffer.

The preselective amplification mixtures prepared by adding 15 μl of AFLP core mix, 0.5 μl of each *Eco*RI and *Mse*I primer (Applied Biosystems) and restriction ligated sample about 4.0 μl so as to have total 20 μl of preselective mixture. This mixture was run on a thermal cycler programmed at 72 °C for 2 min, 94 °C for 20 s, 56 °C for 30 s, 72 °C for 2 min and then 60 °C for 30 min. Final incubation was at 40 °C. The preselective amplified reaction was again diluted 20-fold with TE. Selective amplification was carried out with different combination of *Eco*RI and *Mse*I primers given in (Table 3) (Applied Biosystems). Reaction mixture for selective primer was *Eco*RI (1.0 μl) and *Mse*I (1.0 μl) AFLP core mix 15 μl and preselective mixture diluted 3.0 μl. Selective amplification was carried out in touch down PCR programme. Initial 94 °C for 2 min followed by 10 cycle of 94 °C for 20 s 66 °C (−10 °C/cycle) for 30 s and 72 °C for 2 min and 20 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min with a subsequent hold for 30 min at 60 °C and final incubation at 4 °C. An amplified product (0.5 μl) was mixed with loading buffer (25 μl) and was placed in 48 wells for Genescan Analyzer in 310 AFLP Programmer. About 64 combinations of primers were tried and only 7 combinations generated highest number of peaks. Binary data were obtained after subjecting the Genescanned data to ABI PRISM, Genotype 3.7 NT Software.

3.8. Statistical analysis

The amplified fragments were resolved on Genescan Analysis Software; (version 3.1; Applied Biosystems). Sixty four combinations of *Eco*RI and *Mse*I primers were initially screened by AFLP results obtained from three accessions. The results were used for selecting the primers that relayed maximum genomic polymorphism in *W. somnifera*. The binary data were subjected to UPGMA analysis using NTSYS software. Finally, seven primer combinations which gave distinct dendrograms were selected for detailed AFLP analysis of the DNA extracted from the 15 accessions. For each primer combination, the size of AFLP peaks ranging from 50 to 500 bases were scored for absence (0) or presence (1) across the 15 accessions. The genetic similarity (GS) was estimated between the two markers according to Jaccard's coefficient (Jaccard, 1908). $GS_{ij} = a/a + b + c$, where GS_{ij} is the measure of genetic similarity between individuals I and j, a is the number of polymorphic fragments that are shared by i and j, b is the number of fragments present in i and absent in j, and c is the number of fragments present in j and absent in i. The statistical analysis was carried out by NTSYS-PC software (v.1.7, Rohlf, 1992). A dendrogram was constructed employing UPGMA (unweighted pair grouping method of arithmetic averages) method according to Sneath and Sokal (1973) to group individuals into discrete clusters.

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