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Comprehensive metabolic fingerprinting of *Withania somnifera* leaf and root extracts

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ABSTRACT

Profiling of metabolites is a rapidly expanding area of research for resolving metabolic pathways. Metabolic fingerprinting in medicinally important plants is critical to establishing the quality of herbal medicines. In the present study, metabolic profiling of crude extracts of leaf and root of *Withania somnifera* (Ashwagandha), an important medicinal plant of Indian system of medicine (ISM) was carried out using NMR and chromatographic (HPLC and GC–MS) techniques. A total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots were unambiguously identified. Twenty-nine of these were common to the two tissues. These included fatty acids, organic acids, amino acids, sugars and sterol based compounds. Eleven bioactive sterol–lactone molecules were also identified. Twenty-seven of the identified metabolites were quantified. Highly significant qualitative and quantitative differences were noticed between the leaf and root tissues, particularly with respect to the secondary metabolites.

1. Introduction

Withania somnifera (L.) Dunal (Solanaceae) commonly known as Ashwagandha/Indian ginseng/winter cherry, is one of the most esteemed medicinal plants used in Indian Ayurveda for over 3000 years (Gupta and Rana, 2007; Singh et al., 2001). It is used as herbal medicine in various forms (decoctions, infusions, ointments, powder and syrup) in different parts of the world (Archana and Namasivayam, 1999; Davis and Kuttan, 2001; Kumar et al., 2007), for all age groups of patients without any side effects even during pregnancy (Gupta and Rana, 2007; Sharma et al., 1985). The extracts as well as different isolated bioactive constituents of *W. somnifera* have been reported to possess adaptogenic, anticancer, anti-convulsant, immunomodulatory, antioxidative and neurological effects. The plant is also considered efficacious in the treatment of arthritis, geriatric, behavioural and stress related problems (Dhuley, 2001; Gupta and Rana, 2007; Kaur et al., 2001; Mishra et al., 2000; Ray and Gupta, 1994; Schliebs et al., 1973; Sethi et al., 1970). Several bioactive alkaloids and sterol-lactone based phytochemicals, e.g. ashwagandhine, cuscohygrine, isopelletierine, anaferine, anhygrine, tropine, sitoindosides (sapo-nins), the diversely functionalized withanolides, withanamides, and glycowithanolides have been isolated from different parts of this plant (Matsuda et al., 2001; Mishra et al., 2005, 2008; Rahman et al., 1993, 1999, 2003). Its increasing therapeutic benefits continuously attract the attention of pharmacologists for biomedical investigations on plant extracts and isolated phytochemicals (Bani et al., 2006; Chang et al., 2007; Chen et al., 2008; Kaieh et al., 2007; Malik et al., 2007; Mulabagal et al., 2009; Nair and Jayaprakasam, 2007a,b; Pan et al., 2009).

The metabolic constituents, particularly secondary metabolites differ with the variety of *W. somnifera*, tissue type and sometimes with growth conditions (Abraham et al., 1968). Such variations often lead to inconsistent therapeutic and health promoting properties of various commercial *Withania* preparations (Dhar et al., 2006; Sangwan et al., 2004). This causes difficulties in the compositional standardization of herbal formulations and the commercial exploitation of this plant since in a multi-component therapeutic system, different constituents could influence the health effects through complex multi-target interactions. A recent review (Deocaris et al., 2008) narrates cases where multi-component *W. somnifera* extracts showed better medicinal efficiency than the purified compounds. Hence, instead of tracking a few marker compounds Chaurasiya et al. (2008), comprehensive phytochemical fingerprinting needs to be carried out on the plant material to be used for health benefits (Mohn et al., 2009; Shyur and Yang, 2008; Wang et al., 2005).

The analysis of total metabolome of a plant is important to extend our understanding of complex biochemical processes within a

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plant. Significant technological advances in analytical systems like NMR, GC–MS and HPLC have opened up new avenues for plant metabolomics research aimed at rapidly identifying a large number of metabolites quantitatively and qualitatively. This has become an important area of investigations in pharmacology and functional genomics of medicinal plants. Comprehensive chemical analysis is required not only to establish correlation between complex chemical mixtures and molecular pharmacology but also to understand complex cellular processes and biochemical pathways via metabolite-to-gene network (Nakabayashi et al., 2009). We report the application of various approaches for broad based chemical analysis to identify targeted and non-targeted metabolites in roots and leaves of *W. somnifera* and quantify some of those.

2. Results and discussion

Leaves and root tissues of *W. somnifera* were extracted with *n*-hexane followed by warm (~35 °C) methanol–water (90–70%, MeOH, step wise successively). After liquid–liquid partition of methanolic water portion with $CHCl_3$ followed by *n*-BuOH, metabolites repertoire of the plant was distributed into four fractions of different polarities (*n*-hexane, aqueous-methanol, chloroform and *n*-butanol). Each fraction was then subjected to NMR, GC–MS and sometimes to HPLC–PDA analysis. Metabolic content of leaf and root tissues extracted by solvents of different polarities are presented in Table 1. Quantity of metabolite in leaves was much higher than that in roots, particularly in the aqueous-methanolic fraction.

Table 1
Metabolite content of different fractions from leaf and root of *W. somnifera*.

Extract partition	Total metabolite content mg/gm of DW	
	Leaf	Root
Hexane	34.29 ± 2.0	4.44 ± 0.8
$CHCl_3$	35.71 ± 1.5	10.00 ± 1.0
n-BuOH	28.57 ± 1.6	11.11 ± 1.2
Methanolic water	228.57 ± 5.2	15.00 ± 1.6

2.1. Metabolic analysis of n-hexane extract

Metabolomic analysis of *n*-hexane extract of *W. somnifera* leaves and roots was performed by NMR spectroscopy and GC–MS. ^{1}H NMR spectra of both leaf (Fig. 1) and root (Supplementary Fig. 1) extracts predominantly contained different saturated and unsaturated fatty acids. Signals at δ 1.6 and at δ 2.3 represented the β-CH_2 and α-CH_2 of the fatty acids (Knothe and Kenar, 2004). The signals of all other protons of hydrocarbon chain of fatty acids appeared at δ 1.3. The appearance of olefinic protons at δ 5.35 indicated the presence of unsaturated fatty acids whereas signals at δ 2.07 indicated the allylic protons of unsaturated fatty acid. In addition, the characteristic bis-allylic signals (triplet) of di and tri unsaturated fatty acids appeared at δ 2.8. Homo decoupling experiment of the olefinic protons (δ 5.35) altered the triplet signals of bis-allylic protons into two distinguished singlets at δ 2.77 and δ 2.83, indicating the presence of di and tri unsaturated fatty acids. The 18:3 fatty acids were identified by their characteristic triplet methyl signals at δ 0.99 particularly in the spectrum of leaf *n*-hexane extract. The downfield shift of methyl signals for 18:3 (linole-

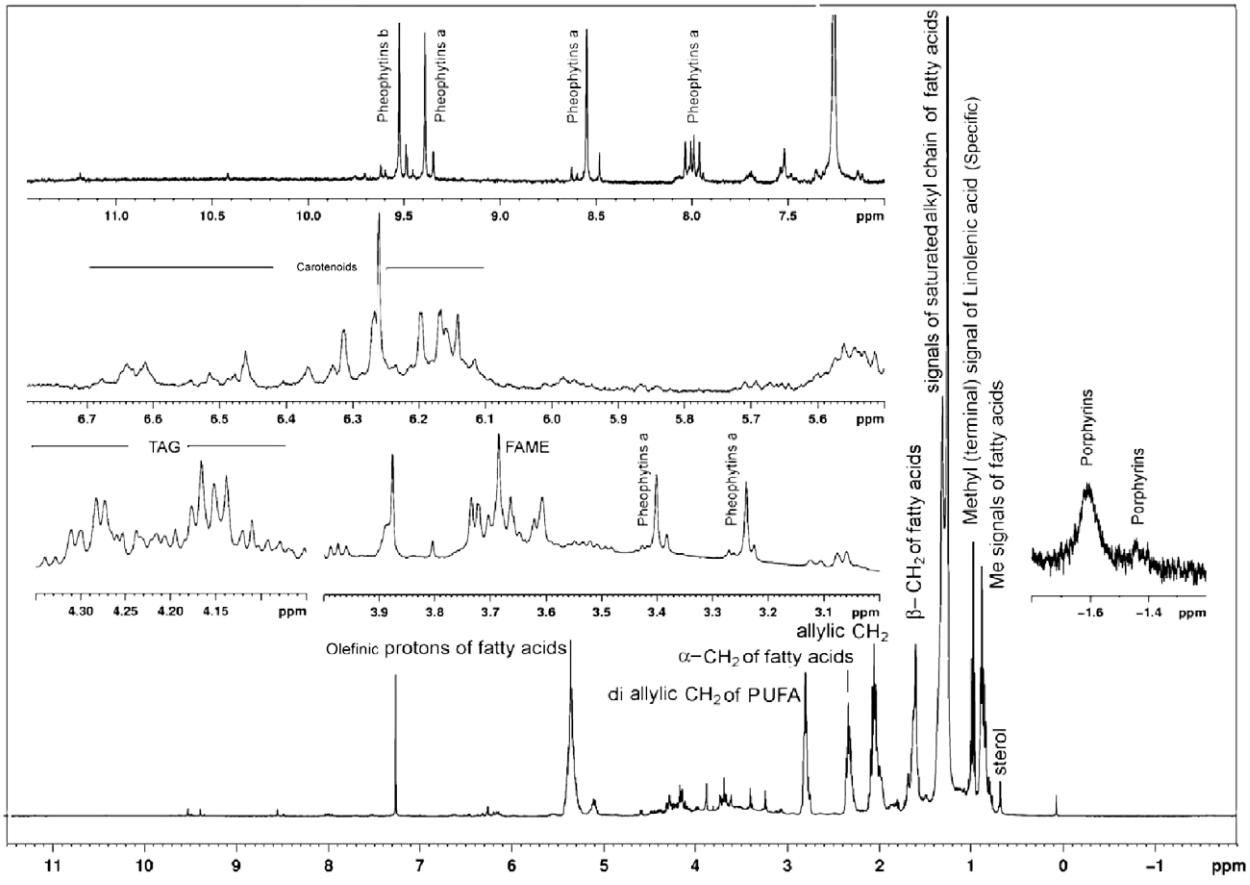

Fig. 1. ^{1}H NMR spectra of the hexane extract of *W. somnifera* leaves.

nic acid) fatty acids occurred due to proximity of the unsaturated double bond (Knothe and Kenar, 2004). Terminal methyl signals of other fatty acids appeared collectively at δ 0.90. In case of root samples, the signals of the terminal methyl were not very distinct due to overlap.

The ^{1}H NMR spectrum of *n*-hexane extract showed characteristic double doublet (dd) signals (δ 4.1–4.3) of sn1 and sn3 protons of triacylglycerol (TAG) (Annarao et al., 2008). Minor amounts of methyl esters of fatty acids were indicated by singlet (for O–Me group) signals at δ 3.6. The characteristic signal of 18-CH_3 group of sterol appeared distinctly at δ 0.7. Several signals at δ 6.0–6.5 might be attributed to the carotenoids (Sobolev et al., 2005). Integration ratio with respect to TSP signals indicated that the carotenoid content of leaf extract was higher than that of root. This is expected, as major role of carotenoids in leaves is to protect leaf from excessive light stress. It was further observed that percentage of TAG was much higher in root extract than in leaves. The presence of two signals in the up field region (δ −1.43 and δ −1.6) of the spectrum of leaves was characteristic for N–H group of the porphyrins (Sobolev et al., 2005). Pheophytins are the degraded products of chlorophylls. During metabolite extraction, the chlorophylls lose their magnesium ions and become pheophytins. The signals of part of phytal fragments (–O–CH_2–CH=C(CH_3)–) of chlorophylls and other part of pheophytins also appeared at δ 9.52, 9.35, 8.5, 8.0, 3.4 and 3.24. The ratio of chlorophylls *a* and *b* was determined as 3:1 by integration of singlet signals at δ 9.37 and δ 9.55. Other minor signals in the range of δ 11–7 may have appeared due to oxidised products of chlorophylls. The observed signals of all the protons and the corresponding ^{13}C signals (identified by ^{1}H–^{13}C HSQC mapping) are presented in Table 2.

To determine the composition of individual fatty acids and sterols, the *n*-hexane extract was subjected to GC–MS analysis after esterification (methyl ester). Palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) were major fatty acids present in the leaf and root samples (Supplementary Fig. 2). These fatty acids belong to membrane lipids of plant tissues. Percentage peak area of the GC chromatograms revealed that palmitic acid and linolenic acid were the predominant fatty acids present in the leaves whereas roots were richer in palmitic acid and linoleic acid. Quantitative data of all major fatty acids in the leaves and roots are documented in Table 3. GC–MS analysis further suggests the presence of many others minor but very long chain fatty acids (Fig. 2) particularly in the root samples, indicating higher activity in the stearoyl-CoA elongation activity in the root tissue as compared to the leaf tissue (Schreiber et al., 2005). Presence of each fatty acid was indicated by respective molecular ions peak in the mass spectrum together with the characteristic peak at *m*/*z* 74 (base peak) that appeared due to McLafferty rearrangement and the peak at *m*/*z* 87 that appeared due to loss of $(CH_2)_2CO_2CH_3^+$. Other respective logically defined mass fragments also appeared in the spectrum. Presence of campesterol and stigmasterol at t_R 53.09 and 53.35 were indicated in the GC–MS analysis, particularly in the *n*-hexane extract of root (Supplementary Fig. 2, Supplementary Table 1).

Table 3
GC–MS identified major metabolites of hexane extract of *W. somnifera*.

Metabolites	t_R (min)	MS data (*m*/*z*)	Peak area (%)	Amount mg/g of DW
Palmitic acid (16:0) (L & R)	30.6	270 (M^+), 87 (58%), 74 (100%), 55 (12%)	10.35 (L) 26.64 (R)	3.55 ± 0.5 (L) 1.18 ± 0.2 (R)
Oleic acid (18:1) (L & R)	34.3	298 (M^+), 87 (60%), 74 (100%), 55 (18%)	2.08 (L) 8.84 (R)	0.71 ± 0.1 (L) 0.39 ± 0.1 (R)
Linoleic acid (18:2) (L & R)	34.6	294 (M^+), 109 (22), 95 (38%), 81 (100%), 67 (72%), 55 (34%)	4.43 (L) 29.52 (R)	1.52 ± 0.2 (L) 1.31 ± 0.2 (R)
Linolenic acid (18:3) (L & R)	35.1	292 (M^+), 121 (20%), 108 (22%), 74 (100%), 55 (24%)	12.74 (L) 3.30 (R)	4.38 ± 0.5 (L) 0.15 ± 0.1 (R)

Leaves (L), roots (R).

2.2. Metabolic analysis of $CHCl_3$ and n-BuOH partition

^{1}H NMR spectrum of *W. somnifera* leaf extract is presented in Fig. 3. The presence of double doublet at δ 6.8 and δ 6.5 together with distinguished doublet signals at δ 6.3 and δ 5.8 indicated the presence of withanolide skeleton. Characteristic singlet signals of methyl series of withanolide frame work were observed in the range of δ 0.6–2.2. Comparison of the spectrum with the purified withanolide standards (Supplementary Figs. 3–5) established the presence of withaferin-A and withanone in the mixture. The ^{13}C signals of each metabolite were recognized by ^{1}H–^{13}C HSQC spectrum (Supplementary Fig. 6a and b) and compared with the literature data of the pure compounds (Tuli and Sangwan, 2009). Both ^{1}H and ^{13}C signals clearly indicated the presence of withaferin-A and withanone as the major metabolite in the $CHCl_3$ partition of leaves. All the related NMR data are presented in Table 4. Branch signals at δ 0.9 of the ^{1}H spectrum appeared due to aliphatic chain of β-sitosterol. GC–MS analysis of this partition indicated the presence of β-sitosterol in this partition. There were some unassigned

Table 2
NMR identified metabolites from hexane extract of *W. somnifera*.

Metabolites	1H Chemical shift δ ppm	13C Chemical shift δ ppm
Porphyrines (L)[a]	−1.43 (bs), −1.60 (bs)	
Carotenoids (L & R)[a]	6.0–6.6	126.9, 127.5, 130.0, 132.5, 132.6
Saturated (L & R)[a]	0.90 (t), 1.27–1.33, 1.60, 2.33	39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5, 24.5, 22.5, 20.8, 14.5179.0
Mono unsaturated (L & R)[a]	0.90 (t), 1.27–1.33, 1.60, 2.07, 2.33, 5.35	39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5, 24.5, 22.5, 20.8, 14.5128.0, 130.0, 179.0
Di-unsaturated (L & R)[a]	0.90 (t), 1.27–1.33, 1.60, 2.07, 2.33, 2.77 (t), 5.35	39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5, 24.5, 22.5, 20.8, 14.5128.0, 130.0, 179.0
Tri & poly-unsaturated (L & R)[a]	0.99 (t), 1.27–1.33, 1.60, 2.07, 2.33, 2.83 (t), 5.35	39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5, 24.5, 22.5, 20.8, 14.5, 128.0, 130.0, 179.0
Fatty acid methyl ester (S) (L & R)[a]	3.5–3.7 (s)	Not detected
Pheophytin a (L)[a]	9.37 (s), 8.55 (s), 8.0 (m), 3.40 (s), 3.24 (s)	Not detected
Pheophytin b (L)[a]	9.55 (s)	Not detected
Sterol (L & R)[a]	0.65 (s)	Not detected
TAG (L & R)[a]	4.1–4.35, 5.05–5.15 (bs)	Not detected

Leaves (L), roots (R).
[a] Identified by NMR.

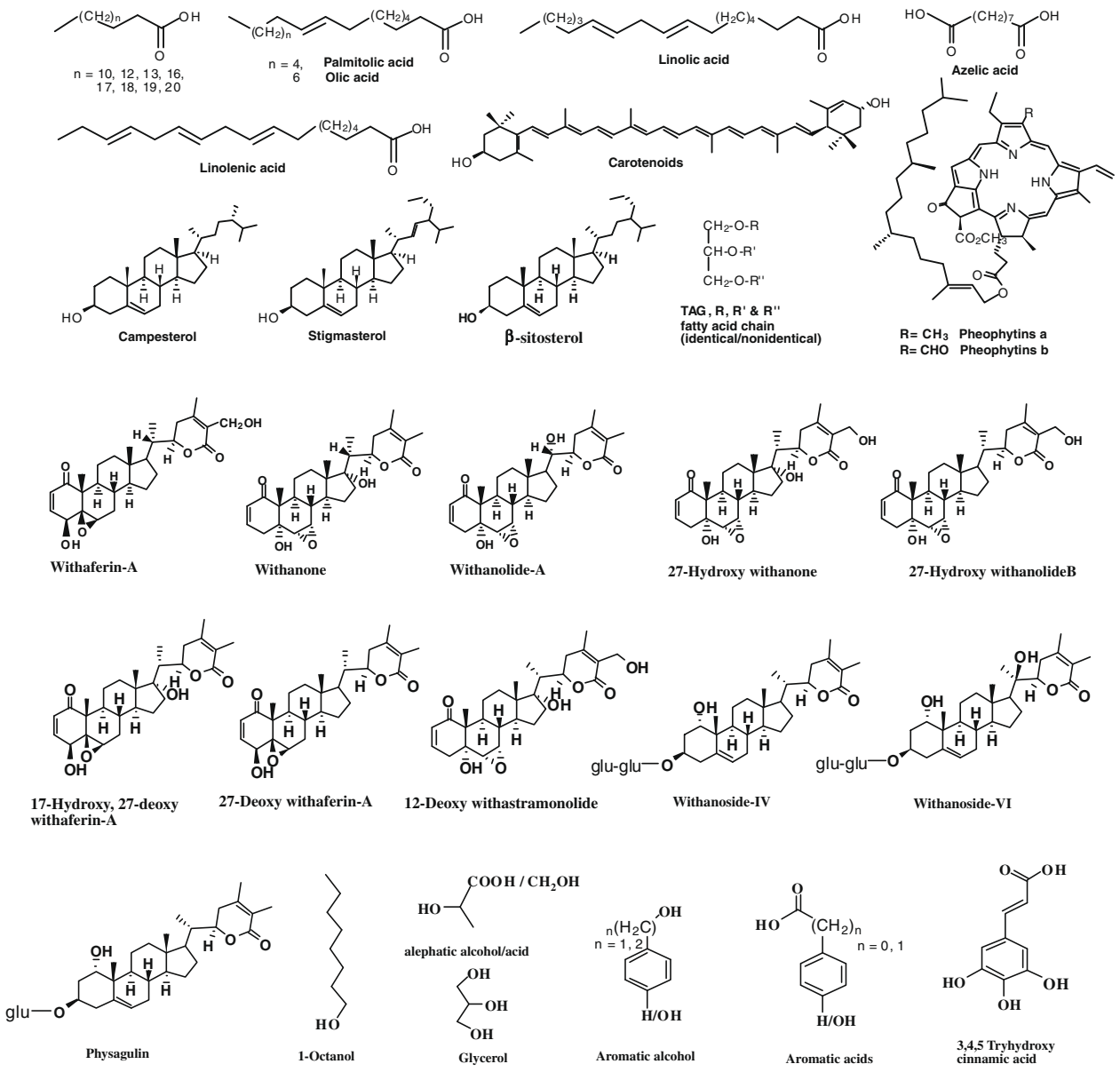

Fig. 2. Some of the phytochemicals identified in *W. sominfera*.

signals appearing in the range of δ 5.2–5.7 of the ^{1}H NMR spectrum which may be due to the presence of minor amounts of withanolides in the mixture. The corresponding ^{1}H NMR spectrum of the root (Supplementary Fig. 7) was not distinct like leaf but it was clear enough to indicate the presence of withanolide skeleton. The ^{1}H signals at δ 6.6 (m), 5.85 (d), 4.2 (m), 1.95 (s), l.85 (s), 1.2 (s), 0.95 (s) and corresponding ^{13}C signals at δ 12.0, 13.0, 14.0, 22.0, 57.0, 129.5140.0 were identified by HSQC spectrum (Supplementary Fig. 8) provided sufficient evidence for the presence of withanolide A. Broadness of the spectrum indicated the presence of significant amounts of similar type of compounds. HPLC analysis of the $CHCl_3$ partition of both leaf and root samples (Supplementary Fig. 9) were performed for further identification of other NMR-non identifiable withanolides. Assignments of resultant chromatograms were performed by matching with the chromatogram of purified withanolides and subsequently by co-chromatography. The analysis suggested that withaferin-A and withanone were the major metabolites present in the leaf as shown by NMR and withanolide A and withanone are major metabolites in the root. Other metabolites detected are 27-hydroxy withanone, 17-hydroxy 27-deoxy withaferin-A, 27-hydroxy withanolide B, 27-deoxy withaferin-A, 12-deoxy withastramonolide. Each of the withanolides was quantified by HPLC using the calibration curve of the standard samples. The qualitative and quantitative data on the metabolites established by NMR and HPLC are presented in the Table 4 and the structures of the identified metabolites are presented in Fig. 2. However, NMR analysis was not enough to provide any useful information about chemical constituents of the *n*-BuOH fraction. The HPLC–PDA analysis (Supplementary Fig. 10) of the *n*-BuOH fraction of leaf is indicating the presence of physagulin, withanoside IV and withanoside VI. However, chromatogram of same fraction of root is cumbersome but reasonable enough to indicate the presence of withanoside IV and withanoside VI. Presence of these compounds was further confirmed by co-chromatography with standard. To explore further the metabolic composition of $CHCl_3$ and *n*-BuOH fractions, GC–MS analysis was carried out. It indicated the presence of 1-octanol, different aromatic alcohols and aromatic acids. Presences of these compounds are logically

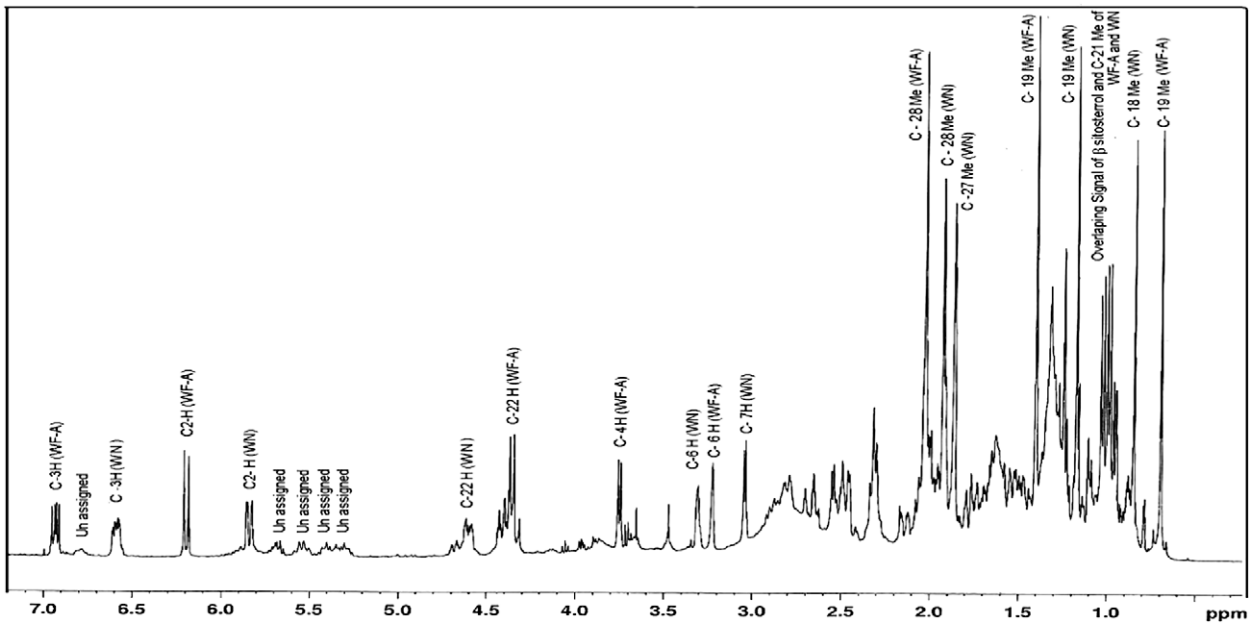

Fig. 3. ^{1}H NMR spectrum of the $CHCl_3$ partition of the *W. somnifera* leaves. WF-A represents withferin-A and WN represent withanone.

Table 4
Identified metabolites in $CHCl_3$ and *n*-BuOH partition of *W. somnifera*.

Metabolites	NMR (δ ppm) and MS/MS (*m*/*z*) data		t_R (min)	*k*′	α	Amount mg/g of DW
	^{1}H Chemical shift δ ppm	^{13}C Chemical shift δ ppm				
$CHCl_3$ fraction						3.45 ± 0.4 (L)[c]
27-Hydroxy withanone (L & R)[b]			16.9 ± 0.5	7.45	1.81	0.50 ± 0.1 (R)[c]
17-Hydroxy, 27-deoxy withaferin-A (L & R)[b]			28.9 ± 0.5	13.45	1.17	3.61 ± 0.5 (L)[c] 0.66 ± 0.2 (R)[c]
Withaferin-A[a,b]						
493 [M+Na$^+$], 471.2, 299.0 (L & R)	6.9 (dd, *J* = 10), 6.21 (d, *J* = 10), 4.3–4.45 (m), 3.75 (d), 3.2 (bs), 2.0 (s), 1.40 (s), 1.20 (d), 0.7 (s)	12.0, 17.5, 20.0, 63.0, 70.0, 74.5, 132.5, 143, 202	33.5 ± 0.5	15.75	1.07	22.31 ± 1.0 (L)[c] 0.92 ± 0.4 (R)[c]
27-Hydroxy withanolide B (L & R)[b]			35.8 ± 0.5	16.9	1.04	2.78 ± 0.5 (L)[c] 0.55 ± 0.2 (R)[c]
Withanolides A 493[M+Na$^+$] (L & R)[a,b]	6.6 (dd, *J* = 10), 5.85 (d, *J* = 10), 4.2 (m), 1.95 (s), l.85 (s), 1.2 (s), 0.95 (s)	12.0, 13.0, 14.0, 22.0, 57.0, 129.5, 140, 202	37.2 ± 0.5	17.6	1.03	2.11 ± 0.5 (L)[c] 3.88 ± 0.7 (R)[c]
Withanone 493 [M+Na$^+$], 431.1263.1 (L & R)[a,b]	6.6 (dd, *J* = 10), 5.85 (d, *J* = 10), 4.6 (m), 3.05 (d), 1.90 (s), 1.85 (s), 1.60 (bm), 1.20 (d), 0.8 (s)	13.0, 14.7, 15.0, 20.5, 56.5, 57.0, 78.0, 129, 140, 203	38.3 ± 0.5	18.15	1.15	18.42 ± 0.8 (L)[c] 5.54 ± 0.4 (R)[c]
12-Deoxy withastromonolide (L & R)[b]			43.8 ± 0.5	20.9	1.14	2.15 ± 0.5 (L)[c] 1.90 ± 0.5 (R)[c]
27-Deoxy withaferin-A (L & R)[b]			49.7 ± 0.5	23.85	–	1.63 ± 0.2 (L)[c] 3.94 ± 0.4 (R)[c]
β-Sitosterol (L)[a]	0.90–1.10 (m)				Not quantified	
n-BuOH fraction						
Withanoside IV (L & R)			26.9 ± 0.5	25.9	1.07	1.60 ± 0.2 (L)[c] 0.44 ± 0.1 (R)[c]
Physagulin (L & R)			28.6 ± 0.5	27.6	1.14	3.46 ± 0.4 (L)[c] Not detected (R)[c]
Withanoside VI (L & R)			32.4 ± 0.5	31.4	–	1.90 ± 0.2 (L)[c] 3.74 ± 0.2 (R)[c]

Leaves (L), roots (R). *k*′ is the capacity factor and α is the separation factor; *k* and α indicates the quality of the HPLC analysis (Sidhu et al., 2003).
[a] Identified by NMR.
[b] HPLC–PDA.
[c] Quantified by HPLC.

supported by their respective mass fragmentation pattern obtained from GC–MS analysis (Table 5).

2.3. Analysis of methanolic water partition

Metabolic profiling of water extract was analyzed mainly by NMR, though in case of root samples, GC–MS was also applied. ^{1}H spectrum of the water extract is presented in Fig. 4. Assignment of the compounds was thoroughly done comparing the ^{1}H spectra of reference compounds together with Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/metabolomics/) and wherever necessary, by spiking with appropriate internal standards. 2D COSY and HSQC spectra were also extensively used to resolve the complexity of the overlapping/interfering spectral regions to identify the exact molecule in the extract.

Table 5
Identified metabolites by GC–MS in $CHCl_3$ and *n*-BuOH partition.

Metabolites	t_R (min)	Molecular formula of the derivative	MS data (*m*/*z*)
2-Hydroxy propanol (L)	3.80 ± 0.3	$C_9H_{24}O_2Si_2$	220 (M^+), 205 (M^+-CH_3, 40%), 147, 117, 73 (Me_3Si, 100%)
2-Hydroxy propanoic acid (L)	6.80 ± 0.3	$C_9H_{22}O_2Si_2$	219 (M^+), 147, 117, 73 (Me_3Si, 100%)
1-Octanol (L & R)	7.11 ± 0.5	$C_{11}H_{26}OSi$	187 (M^+-CH_3, 24%), 147 (20%), 103 (32%), 73 (Me_3Si, 100%)
Glycerol (L)	11.98 ± 0.2	$C_{12}H_{32}O_3Si_3$	293 (M^+-CH_3, 24%), 218, 205, 147, 73 (Me_3Si, 100%)
Benzyl alcohol (R)	12.14 ± 0.1	$C_{10}H_{16}OSi$	180 (M^+, 12%), 165 (M^+-CH_3, 100%), 135 (M^+-3CH_3, 42%), 91($PhCH_2$, 64%), 73 (Me_3Si, 60%)
2-Phenyl ethanol (R)	14.21 ± 0.3	$C_{11}H_{18}OSi$	180 (M^+, 12%), 179 ($M^+-CH_3^{\pm}$, 54%), 105 (Ph-$CH_2CH_2^{\pm}$, 20%), 73 (Me_3Si, 100%)
Benzoic acid (L & R)	15.81 ± 0.5	$C_{10}H_{14}O_2Si$	194 (M^+), 179 (M^+-CH_3, 100%), 135 (34%), 105 ($PhCO^+$, 40%), 77 (Ph^+, 46%), 75 (18%)
Butandioic acid (L)	16.14 ± 0.3	$C_{10}H_{22}O_4Si_2$	247 (M^+-CH_3, 12%), 147, 73 (Me_3Si, 100%)
Phenyl acetic acid (L & R)	17.63 ± 0.5	$C_{11}H_{16}O_2Si$	208 (M^+), 193 ($M-CH_3$, 12%), 164 ($M-2CH_3$, 22%), 91 ($PhCH_2^+$, 10%), 73 (Me_3Si, 100%)
p-Hydroxy, phenyl ethanol (L)	22.44 ± 0.5	$C_{14}H_{26}O_2Si_2$	282 (M^+), 223, 179 (100%), 73 (Me_3Si, 45%)
p-Hydroxy benzoic acid (L)	24.24 ± 0.5	$C_{13}H_{22}O_3Si_2$	282 (M^+, 26%), 267 (M^+-2CH_3, 88%), 223 (M^+-Me_3Si, 84%), 193 (80%), 126 (26%), 73 (Me_3Si, 100%)
p-Hydroxy, phenyl acetic acid (R)	24.57 ± 0.0	$C_{12}H_{18}O_3Si_2$	238 (M^+, 36%), 179 ($M^+-Me_3Si-CH_3$, 100%), 163 ($M^+-Me_3SiO-3Me_3$, 34%), 73 (Me_3Si, 42%)
3,4,5-Trihydroxy cinnamic acid (R)	37.17 ± 0.5	$C_{15}H_{22}O_5Si$	310 (M^+, 68%), 280 (M^+-2CH_3, 100%), 249 (12%), 73 (Me_3Si, 68%)
β-Sitosterol (L)	51.91 ± 0.5	$C_{32}H_{58}OSi$	488 (MH^+), 396, 357, 129, 75, 44

Leaves (L), roots (R): some of the molecules may appear in either of the fractions depending upon the ratio of the solvent used during partitioning.

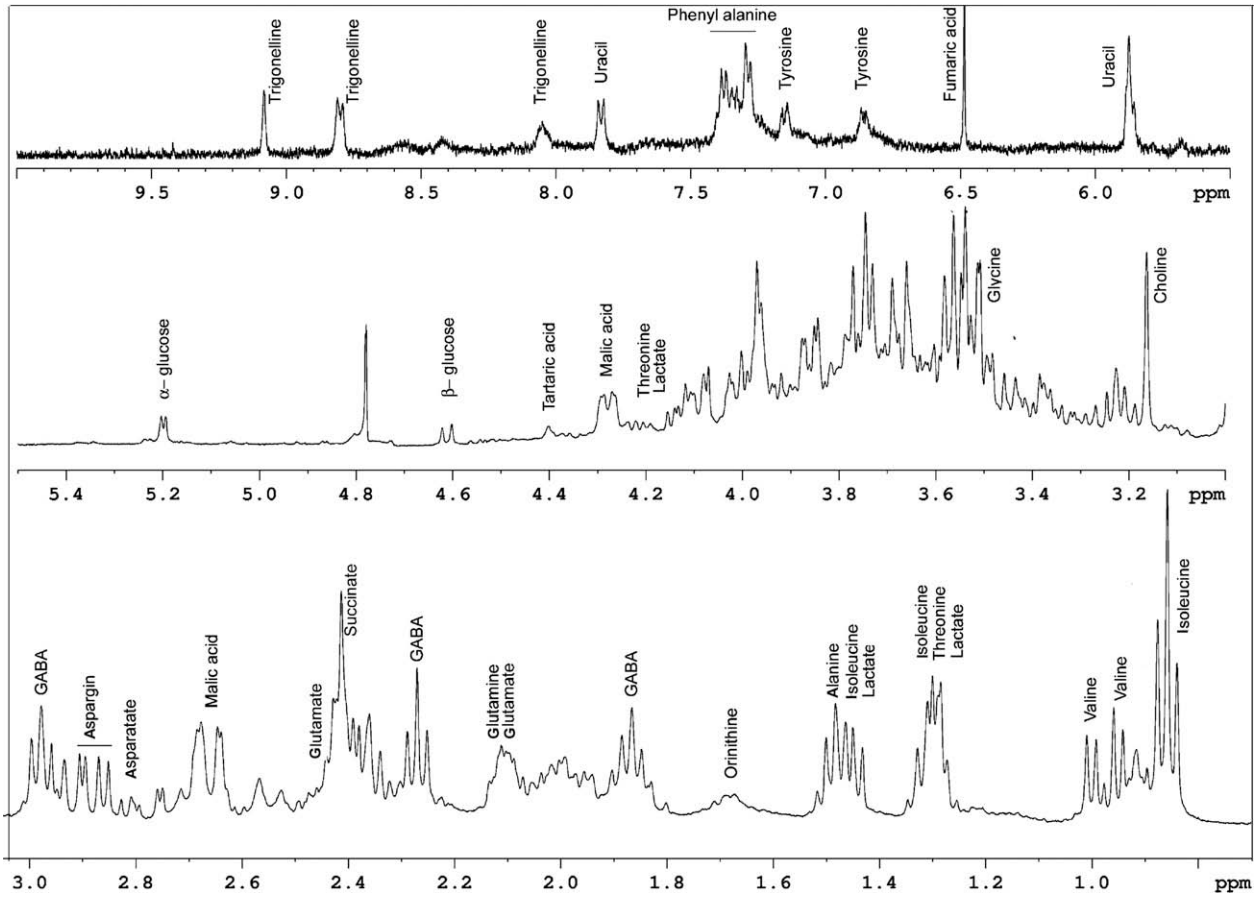

Fig. 4. ^{1}H NMR spectrum of the aqueous fraction of *W. somnifera* leaves.

The entire ^{1}H spectrum of aqueous fraction may be divided into three major regions. δ 0.0–3.5 region is rich with amino acids. δ 3.5–5.5 contains sugars and rest of the spectrum is dominated by aromatic compounds. In the ^{1}H NMR spectrum of the leaves, the region of amino acids started with distinct triplet signals of isoleucine, followed by two sharp doublets of valine. The doublet signals of two δ-CH_3 (0.95, 0.96) leucine were detected by enlarging the spectral segment using Bruker X-win NMR software. Multiplet signals of γ-CH_2 of isoleucine appeared at δ 1.35 and it also shows distinct COSY interactions with δ-CH_3 of isoleucine. Complex multiplet signal at δ 1.48 was observed due to overlapping signals of β-CH_3 of alanine (doublet) and multiplet signals of γ-CH_2 of isoleucine. Branch broad multiplet signals at δ 1.6–1.8 may be due to the presence of ornithine in the extract (Lee et al., 2009). The presence of GABA in the mixture was indicated by characteristics signals at δ 1.90 (m, β-CH_2), 2.29 (t, α-CH_2), 3.01 (t, γ-CH_2) and then distinct correlation in COSY spectrum. Characteristic dd signals of malic acid appeared at δ 2.47–2.68 (geminal protons) and it showed the expected correlation with dd signals of neighbouring protons at δ 4.34 (H attached with C–OH). Respective carbon signal appeared at δ 43.5 and δ 71.5. The dd signal at δ 2.68–2.79 is indicative of β-CH_2 of aspartate whereas similar signals at δ 2.85–2.90 suggested the presence of asparagine. Strong singlet signal at δ 2.40 was the signature of succinic acid (Sobolev et al., 2005). The presence of glutamine and glutamate in the water part was identified mainly by COSY cross peak. Overlapping signals of associated protons appeared in regions at δ 2.0–2.44. Strong singlet signal at δ 3.2 is indicative of $N(Me_3)_3$ of choline in the extract. Related carbon signals appeared at δ 55.1 and δ 75.3. The 1D spectral regions at δ 3.2–4.2 of the carbohydrate region is highly congested. It was very difficult to identify any particular signals but all the carbon signals related to carbohydrate skeleton were relatively distinct (δ 63.0, 73.0, 74.2, 76.6, 78.2 and 96.9) in the HSQC spectrum. Overall nature of the spectrum suggested the presence of high amount of sugars in the

Table 6
Identified metabolites from aqueous fraction of *W. somnifera*.

Metabolites	NMR and MS data		t_R (min)	Amount mg/g of DW
	^{1}H δ ppm	^{13}C δ ppm		
Alanine (L)[a]	1.48, 3.77 (q)	17.9, 53.0		Detected
Aspartate (L)[a]	2.68–2.79 (dd), 3.88	36.3		Detected
Asparagine (L & R)[a]	2.85–2.90 (dd), 3.90	36.5		Detected
Choline (L & R)[a]	3.19 (s), 3.52	55.1, 75.3		3.52 ± 0.5
Citric acid (L)[a]	2.58 (m)	46.2, 76.6, 180.6, 182.9		Detected
Fructose-5 TMS (R)[b]	*m/z* 437 (38%), 217 (30%), 204 (68%), 147 (30%), 103, 73 (Me_3Si, 100%)		23.73	Detected
Fructose-5 TMS, $MeOX_1$ I (R)[b]	*m/z* 307 (72%), 217 (62%), 147, 103, 73 (Me_3Si, 100%))		25.68	Detected
Fructose-5 TMS, $MeOX_1$ II (R)[b]	*m/z* 307 (72%), 277, 217 (62%), 147, 103, 73 (Me_3Si, 100%)		26.03	Detected
Fumaric acid (L)[a]	6.52 (s)	135.9		0.6 ± 0.2
GABA (L & R)[a]	1.91(m), 2.29(t), 3.01	41.0		16.74 ± 0.8 (L)
GABA-N,N-TMS, O-TMS $C_{13}H_{33}NO_2Si_3$[b]	*m/z* 319 (M^+, 100%), 304 (M^+-CH_3), 174, 147, 73 (Me_3Si, 88%)		19.79	Detected (R)
Galactose (L & R) $C_2H_{55}NO_6Si_5$[b]	*m/z* 554 (M^+-CH_3), 319 (100%), 217, 205 (28%), 147, 73 (Me_3Si, 88%)		26.24	Detected
Glycerol (R) $C_{12}H_{32}O_3Si_3$[b]	*m/z* 293 ($M-CH_3$), 218 (20%), 205 (40%), 147 (46%), 117 (34%), 73 (Me_3Si, 100%)		12.00	Detected
Glutamate (L & R)[a]	2.06, 2.11, 2.36			Detected
Glutamic acid N-TMS, 2 O-TMS[b] $C_{14}H_{33}NO_4Si_3$	*m/z* 363 (M^+), 348 (M^+-CH_3,12%), 246 (100%), 147, 128 (22%), 73 (Me_3Si, 49%)		22.79	
Glutamine (L & R)[a]	2.14, 2.44, 3.79			Detected
α-Glucose (L & R)[a,b]	4.64 (d, *J* = 3.7)	63.3, 73.0, 74.2, 76.6, 78.2, 93.1		6.11 ± 0.5 (L)
β-Glucose (L & R)[a,b]	5.20 (d, *J* = 7.8)	63.3, 73.0, 74.2, 76.6, 78.2, 96.9		10.22 ± 0.9 (L)
Glycine (L)[a]	3.57 (s)			Detected
Myo-inositol (L)[b]	612 (M^+), 320 (M^+-4TMS), 305($M^+-4TMS-Me$), 217 $M^+-5TMS-2Me$), 147, 73 (Me_3Si, 49%)		28.84	
Isoleucine (L)[a]	0.88 (t, *J* = 7.6), 1.96 (m)			19.83 ± 0.8
Lactic acid (L)[a]	1.33, 4.11			Detected
Lysine (L)[a]	1.47, 1.72, 1.88, 3.02, 3.76	22.5		Detected
Leucine (L)[a]	0.96 (d), 1.69, 3.72			Detected
Succinate (L & R)[a]	2.68–2.79 (dd), 4.31 (dd)	71.5, 43.5		
Malic acid 3 TMS $C_{13}H_{30}O_5Si_3$[b]	*m/z* 335 (M^+-CH_3), 245, 233, 147, 73 (Me_3Si, 100%)		19.64	Detected
N-Acetyl Glucosamine (L) $C_{21}H_{50}N_2O_6Si_4$[b]	*m/z* 538 (M^+), 450, 348, 147, 73 (Me_3Si, 100%)		27.02	
Orinithine (L)[a]	1.6–1.8 (bm)			21.5 ± 0.8
Phenyl alanine (L)[a]	7.3–7.45 (bm)			Detected
Succinate (L)[a]	2.40 (s)			12.75 ± 0.5
Tartaric acid (L)[a]	4.38 (s)			4.10 ± 0.4
Tyrosine (L)[a]	3.08, 3.17, 3.94, 6.88 (d), 7.2 (d)			Detected
Threonine (L)[a]	1.32 (d)	21.92		Detected
Trigonelline (L)[a]	9.1 (s), 8.8, 8.1			1.33 ± 0.3
Uracil (L)[a]	5.95 (s), 7.75 (m),			3.90 ± 0.2
Valine (L)[a]	0.98 (d, *J* = 7.0), 1.04 (d, *J* = 7.0), 2.25 (m)	18, 19		5.60 ± 0.5

Leaves (L), roots (R).
[a] Identified by NMR.
[b] Identified by GC–MS.

extract. However, the presence of α and β anomers of glucose were clearly identified by their respective doublet signals (β; δ 4.61, J = 7.9 Hz; α; δ 5.2, J = 3.8 Hz). GC–MS analysis further indicated presence of other sugars, *i.e.* galactose, N-acetyl glucosamine and myo-inositiol in the extract. The singlet signals at δ 4.4 appeared due to presence of tartaric acid (Sobolev et al., 2005). ^{1}H signals of threonine and lactate generally appeared side by side at δ 1.33 and δ 4.2 due to their low abundance in the extract. The characteristic signals were not clearly observed in the 1D spectrum but in the 2D COSY spectrum they appeared distinctly. The respective carbon peak appeared at δ 22.5. Strong singlet signals at δ 5.8 indicated one of the olefinic protons of uracil and showed clear cross peak at δ 7.85 corresponding to olefinic protons signals. Sharp singlet around δ 6.5 represented fumaric acid in the extract. Signals at δ 6.88 and δ 7.18 were assigned to tyrosine, which was supported by COSY experiments. The branch spectral band from δ 7.3 to δ 7.45 regions and corresponding COSY analysis suggested the occurrence of phenyl alanine. The signatures of trigonelline were observed at δ 8.1, 8.8 and 9.1 ppm. Detailed COSY relations of the individual metabolite are presented as supplementary information (Supplementary Fig. 11a and 11b, Table 2).

^{1}H spectral complexity (overlapping signals) did not allow quantification of all the metabolites. However, a number of them were quantified by integrating the distinct characteristic signals of each metabolite with respect to signal intensity of quantified amount of TSP. NMR spectroscopy of the aqueous aliquots of root samples was not distinctly informative as very high concentration of sugar in this fraction masked other minor signals. However, GC–MS analysis of the extract of root samples indicated the presence of higher amounts of fructose, galactose, glucose and glycerol besides some minor amino acids. Summary of the quantitative and qualitative outcome is presented in Table 6.

3. Concluding remarks

Metabolomic fingerprinting of herbal extracts is desirable to standardise drugs and to establish the scientific basis of their pharmacological action. This study recruited 1D and 2D NMR, HPLC–PDA, GC–MS techniques for rapid metabolome analysis of *Withania* leaf and root extracts. Such analysis is desirable for developing herbal drugs and establishing association with their action through functional genomics and molecular pharmacology. Such knowledge will evolve directions for genetic improvement of medicinal plants for the enhancement of pathways leading to the biosynthesis of bioactive molecules. A total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots were unambiguously identified in *W. somnifera* in this investigation. Twenty-seven of the identified metabolites were quantified. Significant qualitative as well as quantitative differences between the leaf and root tissue, particularly with respect to secondary metabolites were noticed. High resolution NMR like 800/1000 MHz or high resolution instruments like Fourier Transform Ion Cyclotron Resonance Mass Spectrometer can resolve even higher number of molecules and establish those quantitatively in different plant parts. Such a wide metabolomic analyses not only can help set discrete parameters for better defining and quality control of herbal extract but could also serve as diagnostics for their true identity and adulteration with other plants or non-usual parts of the same plant.

4. Experimental

4.1. Plant samples and chemicals

Fresh leaves and roots of *W. somnifera* genotype NMITLI-101 were collected at early flowering stage from 50 different plants of the same age grown in similar conditions at NBRI experimental farm. Collected leaves were dried in shade on blotting papers until constant weight. Dried leaves and roots were ground to powder using grinder mill. All the solvents used for the extraction of phytochemicals from plant tissues were purchased from Qualigen (ExcealR). All deuterated solvents for NMR were purchased from Sigma Chemical Company (USA). All the solvents used for HPLC were of HPLC grade from E. Merck.

4.2. Extraction of metabolites

The dried plant material was extracted with ten times weight of warm (~35 °C) hexane. The solvent portion was collected by filtration and this procedure was repeated five more times until the hexane layer became almost colourless. Separated solvent layer was concentrated under reduced pressure. The resulting sticky mass was stored at −20 °C until analyzed. The remaining solid plant material was further extracted thrice with fivefold excess (w/w) of 90% and then with 70% warm methanol–water. Volume of the extract was reduced to 1/3rd using rotavapour and defatted with equal volume of hexane. Defatted water–methanol layer was partitioned (liquid–liquid) with equal volume of $CHCl_3$ (five times) followed by *n*-BuOH (five times). Each of the layers was dried separately over sodium sulphate, concentrated to semisolid mass and stored at −20 °C till further analysis. Remaining methanolic water layer was lyophilized to dryness and the resulting solid was again saved for analysis.

4.3. NMR analysis

^{1}H NMR spectra of the hexane and aqueous-methanolic extracts were obtained on Bruker Biospin Avance 400 MHz NMR spectrometer using a 5 mm broad band inverse probe head, equipped with shielded z-gradient accessories. 1D ^{1}H NMR spectral analyses of hexane extracts were carried out using one-pulse sequence by dissolving samples in 500 µl deuterated chloroform taken in 5-mm NMR tubes. A reusable sealed capillary tube containing 30 µl of 0.375% of TSP in deuterium oxide was inserted into the NMR tube before recording the spectra. TSP served as chemical shift reference as well as internal standard for quantitative estimation. ^{1}H NMR experiments were also performed with homonuclear decoupling to olefinic CH=CH protons. Typical parameters for both the extractions were: spectral width: 6000 Hz; time domain data points: 32 K; For quantification purpose the effective flip angle of 45° was used, optimized and standardized instead of 90° using total relaxation delay of 7.73 s for complete recovery of the magnetization by taking consideration of our earlier studies on amino acids (Bharti et al., 2008) so that the quantified results are precise; spectrum size: 32 K points; and line broadening for exponential window function: 0.3 Hz. To confirm the assignments, two-dimensional (2D) correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear single quantum correlation (HSQC) were carried out using the Bruker's standard pulse program library. The spectral widths of COSY were 6000 Hz in both dimensions, and 512 t_1 increments for each t_1. Sixteen transients using 2.5 s relaxation delays were added with 2048 complex data. The phase-sensitive data were obtained by the time proportional phase incrementation (TPPI) method. The resulting data were zero-filled up to 1024 in t_1 dimension and were weighted with 90° squared sine window functions in both dimensions prior to double Fourier transformation. Heteronuclear 2D ^{1}H–^{13}C chemical shift correlations were measured using gradient HSQC with a gradient ratio of GPZ1:GPZ2 as 80:20. The experiments were performed with a spectral width of 6000 Hz in F_2 dimension and 24,000 Hz in F_1

dimension, 400 t_1 increments. For each t_1, 96 transients using 1.5 s relaxation delay was added with 2048 complex data points.

4.4. GC–MS analysis

GC–MS analysis was performed using Thermo Trace GC Ultra coupled with Thermo fisher DSQ II mass spectrometers with electron impact ionisation at 70 eV to generate mass spectra. 30 m × 0.25 mm Thermo TR50 column (polysiloxane column coated with 50% methyl and 50% phenyl groups) was used for chromatographic separation of metabolites. To prepare the sample for GC–MS analysis of non-polar hexane extract, 10 mg portion was heated at 60 °C for 6 h with 5 ml of methanolic sulphuric acid (5%, v/v). After cooling, the reaction mixture was diluted and vigorously shaken with 25 ml hexane and 20 ml water. Separated hexane layer was washed with 20 ml water containing 5% (w/v) sodium bicarbonate followed by equal volume of 5% (w/v) sodium chloride solution. Hexane layer was collected and concentrated using rota vapour after drying over anhydrous sodium sulphate. Resulting oily mass was dissolved in 1 ml of GC-grade *n*-hexane and 0.4 μl of the solution subjected to analysis on GC. With an initial 5-min solvent delay time at 70 °C, the oven temperature was increased to 330 °C at 5 °C/min, 5 min isocratic and cooled down to 70 °C followed by an additional 5-min delay. Helium flow was maintained at 1 ml/min and split ratio was maintained 1/60. The resulting GC–MS profile was analyzed using WILLY and NIST mass spectral library and by matching the chromatogram with supelco FAME (fatty acid methyl ester) mixture and whenever is possible, with appropriated standards. For the GC–MS analysis of other than hexane extracts, the TMS derivative of the sample was prepared. Approximately 5 mg of the sample was suspended in 40 μl of the solution of methoxylamine hydrochloride in pyridine (20 mg/ml). The mixture was shaken for 4 h at 37 °C before adding 70 μl of the 2,2,2-trifluoro-N-methyl-N-trimethylsilyl-acetamide (MSTFA). Shaking was continued for another 30 min. Subsequently, 40 μl of the derivatized solution was sampled with 20 μl of *n*-hexane. The GC–MS running conditions were same as mentioned earlier. Quantification of metabolite was done using its percentage peak area appeared at the total ion chromatogram in GC–MS analysis.

4.5. HPLC analysis of the samples

HPLC–PDA analysis of $CHCl_3$ fraction was performed on the system from waters (Milford, MA, USA). The separation was carried out using waters reverse phase column (3.9 × 150 mm, 5 μm) and binary gradient elution. The two solvents used for the analysis consisted of water containing 0.1% acetic acid (solvent A) and methanol containing 0.1% acetic acid (solvent B). Gradient programming of the solvent system was carried out at 27 °C, initially at 60% A changed to 40% A at 30 min, maintained for the next 2.0 min, changed to 25% A at 45 min and then to 5% A at 54 min at flow rate of 0.6 ml/min and then at a flow rate of 1.0 ml/min. The mobile phase was changed to 0% A at 55 min. The solvent composition was maintained until the run time reached 60 min. All the gradient segments were linear (curve type 6). The wavelength scan range of the PDA was set to 190–350 nm. The chromatograms were recorded at 227 nm. HPLC–PDA analysis of *n*-BuOH fraction was carried out using the protocol-I (acetonitrile: water gradient) of Malik et al. (2007) for glycosylated withanolides using waters reverse phase column (3.9 × 250 mm, 5 μm). Quantification of withanolides was carried out as by using the peak area of the sample chromatogram in the regression equation of the calibration curve for each withnaolides and glyco-withanolides standard.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.04.001.

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