Metabolic Alterations of *Withania somnifera* (L.) Dunal Fruits at Different Developmental Stages by NMR Spectroscopy

O.P. Sidhu, Sanjay Annarao, Sandipan Chatterjee, Rakesh Tuli, Raja Roy and C.L. Khetrapal

**ABSTRACT:**

Introduction – *Withania somnifera* (*Ashwagandha*) is a high-value Ayurvedic medicinal plant and an important constituent of several dietary supplements. In order to substantiate the health claims, the herb has drawn considerable scientific attention. Objective – The objective of the study was to investigate the alterations in primary and secondary metabolites of *W. somnifera* fruits during its maturity using NMR spectroscopy.

Methodology – Fruits at different stages of development from one week after fertilisation until maturity, classified in seven developmental stages, were analysed by a combined use of one- and two-dimensional NMR experiments.

Results – Seventeen metabolites were characterised and quantified from non-polar and polar extracts of different fruit development stages of *W. somnifera*. The principal component analysis of polar metabolites at different stages could be grossly classified into three metabolic phases, viz. initial phase, developmental phase and maturation phase.

Conclusion – Qualitative and quantitative analysis of metabolites in *W. somnifera* fruits indicated specific stages when fruits can be harvested for obtaining substantial bioactive ingredients for desirable pharmacological activity. This study potentially provides a complementary tool for quality control of herbal medicinal products when *W. somnifera* fruits are used. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: NMR; metabolite profile; fruit development; *Withania somnifera*

**Introduction**

*Withania somnifera* (L.) Dunal (Family Solanaceae), commonly known as *Ashwagandha*, is one of the most valued medicinal plants with a number of pharmaceutical and nutraceutical applications (Anonymous, 1962). The ethnopharmacological properties of this plant include adaptogenic, anti-sedative and anti-convulsion activities (Budhiraja et al., 2000; Mishra et al., 2000). The plant is recommended in many Ayurvedic recipes and has been employed in the treatment of neurological disorders, geriatric debilities, arthritis and stress-related problems (Gupta and Rana, 2007). Several modern molecular pharmacological studies have demonstrated linkage of these therapeutic actions to one or more withanolides present in the herb (Mishra et al., 2000; Ichikawa et al., 2006; Kaileh et al., 2007). Phytochemically, the plant is unique since it possesses diversified sets of active alkaloids and steroidal lactone-based chemical constituents such as ashwagandhine, cuscohygrine, isopelletierine, anaferine, anhygrine, tropine, sitoindoside (saponins), withanone, several withanolide(s), withaferine(s), withanamide(s), physagullin(s) and glycowithanolide(s) in different parts of this plant (Gupta and Rana, 2007; Mirjalili et al., 2009). Withanamides present in this species have been reported to have efficient lipid peroxidation inhibitory activity and potential to prevent progression of Alzheimer’s disorders (Jayaprakasam et al., 2004, 2009). Recently, 79 major and minor primary and secondary metabolites have been characterised from vegetative parts of this plant using NMR and chromatographic techniques (Chatterjee et al., 2010). Very few studies have been carried out on chemical profiling of fruits except that of Abou-Dough (2002) who reported presence of withanolides apart from coumarins, while Jayaprakasam et al. (2004) reported both withanolides and withanamides in mature fruits.

Herbal medicinal extract is a multi-component therapeutic system in which different constituents could influence the health effect through complex and multi targeted interactions (Wang et al., 2005). The target metabolite may not be present in all plant parts. Variation of phytochemicals in different plant parts during their developmental stages may constitute a serious obstacle for the quality control of herbal formulation, which could lead to inconsistent medicinal properties. Hence, comprehensive metabolic content analysis of a plant and its parts is a prerequisite for any herbal formulation. Rapid and reliable fingerprinting and quantification of phytochemicals without separation of individual components therapeutic action is one or more withanolides present in the herb (Mishra et al., 2000; Ichikawa et al., 2006; Kaileh et al., 2007). Phytochemically, the plant is unique since it possesses diversified sets of active alkaloids and steroidal lactone-based chemical constituents such as ashwagandhine, cuscohygrine, isopelletierine, anaferine, anhygrine, tropine, sitoindoside (saponins), withanone, several withanolide(s), withaferine(s), withanamide(s), physagullin(s) and glycowithanolide(s) in different parts of this plant (Gupta and Rana, 2007; Mirjalili et al., 2009). Withanamides present in this species have been reported to have efficient lipid peroxidation inhibitory activity and potential to prevent progression of Alzheimer’s disorders (Jayaprakasam et al., 2004, 2009). Recently, 79 major and minor primary and secondary metabolites have been characterised from vegetative parts of this plant using NMR and chromatographic techniques (Chatterjee et al., 2010). Very few studies have been carried out on chemical profiling of fruits except that of Abou-Dough (2002) who reported presence of withanolides apart from coumarins, while Jayaprakasam et al. (2004) reported both withanolides and withanamides in mature fruits.

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constituents using high-throughput NMR has found an increasing application in metabolomics and chemotaxonomy of the plant species (Sobolev et al., 2005). In the present study, we have found that NMR spectroscopy can be utilised to investigate alterations in the levels of primary and secondary metabolites of W. somnifera and monitor changes in the growth and development of its fruits. The study will also be useful to identify appropriate stages of fruit development for harvesting in order to optimise target metabolites for specific pharmacological applications.

Experimental

Sample collection

Fruits of W. somnifera variety RSS-8, maintained at the experimental research field of the National Botanical Research Institute Lucknow, India, were collected at various stages of development, from fertilised ovule to maturity. The first stage was collected 7 days after fertilisation. Fruits were then collected at an interval of 5 days until maturity. Seven such stages (stages I–VII) were thus collected. Average fresh weight of fruits at stages I–VII was found to be 16.2 ± 1.7, 39.8 ± 2.9, 71.3 ± 1.4, 112.6 ± 4.4, 115.4 ± 3.9, 149.7 ± 4.6 and 168.2 ± 7.2 mg, respectively.

Metabolite extraction

Three replicates of each of the seven stages of fruit development were dried to constant weight using freeze dryer (Heto LyoPro 6000). Samples of dried fruits (5 g) were extracted with hexane (3 × 100 mL) using a tissue homogeniser (Kinematica Polytron Homogenizer PT 6100). Combined extracts were filtered and concentrated to dryness under reduced pressure using a rotary evaporator (Laborota 4000, Heidolph, Germany) and kept for the analysis of non-polar part. The residue from the filtration was air-dried and extracted (3 × 100 mL MeOH/D2O, 4:1) in a manner similar to that of hexane extraction. The combined aqueous methanolic extracts were filtered, concentrated under reduced pressure, lyophilised to remove moisture and kept at −20°C until NMR analysis.

NMR spectroscopy

1H NMR spectra of the hexane and aqueous methanolic extracts were recorded using a Bruker BioSpin Avance 400 MHz NMR spectrometer with a 5 mm broad band inverse probe head, equipped with shielded z-gradient. To facilitate the assignment of the peaks due to individual acids, 1H NMR spectra of pure stearic, palmitic, oleic, linoleic and linolenic acids were also recorded.

One-dimensional 1H−NMR experiments of hexane extracts were obtained by using a one-pulse sequence following the method of Annarao et al. (2008). In each experiment, sample (20 mg) was dissolved in CDCl3 (500 μL) in a 5 mm NMR tube. 1H−NMR experiments were also performed with homonuclear decoupling to the olefinic CH&dbond;CH protons.

Quantitation of metabolites from aqueous methanolic extracts of seven fruit developmental stages was carried out using 1H NMR single-pulse sequence with suppression of the water signal. A sample (20 mg) of dried extract of each stage (in triplicate) was dissolved in MeOD/D2O mixture (500 μL) in a ratio of 4:1 and transferred in a 5 mm NMR tube. A coaxial sealed capillary tube containing D2O (30 μL) with 0.375% (w/v) trimethylsilylpropionate (TSP) was inserted into the NMR tube for NMR measurements. TSP served as a chemical shift reference as well as external standard for quantitative estimation. The spectral width used was 6000 Hz with time domain data points of 32 K. The flip angle of the radiofrequency pulse was 45°, with a relaxation delay of 7.73 s to ensure maximum recovery of the magnetisation to equilibrium between the scans. Typically, 128 scans were accumulated for each sample, and the resulting data were Fourier-transformed after multiplication by the exponential window function using a line-broadening function of 0.3 Hz. Quantitative errors were ±5% based on a gravimetric method previously reported (Bharti et al., 2008) where standard solutions of amino acids were analysed.

To confirm the assignments two-dimensional (2D) phase-sensitive double quantum filtered correlation spectroscopy (COSYDQF), total correlation spectroscopy (TOCSY) and 1H−13C hetero nuclear single quantum correlation (HSQC) experiments were carried out using the Bruker’s standard pulse program library. The spectral widths of COSY were 6000 Hz in both dimensions and 512 t1 increments for each t1; 16 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 the t1 dimension and were weighted with 90° squashed sine window functions in both dimensions prior to double Fourier transformation. In the case of TOCSY, the spin-locking time was set to 60 ms, which included a duration of 2.5 ms for the trim pulses. Heteronuclear two-dimensional 1H−13C chemical shift correlations were measured using gradient HSQC with a gradient ratio of GPZ1-GPZ2 as 80:20:1; the experiments were performed with a spectral width of 6000 Hz in F2 dimension, 24000 Hz in F1 dimension and 400 t1 increments. For each t1, 96 transients were added with 2048 complex data points. The data were weighted with 90°-shifted squashed sine window function in F1 and F2 dimensions before double Fourier transformation.

Data analysis

The proton NMR spectra of polar extracts were individually analysed using AMIX software (version 3.7.10, Bruker BioSpin, Switzerland) capable of performing chemometric multivariate unsupervised and supervised statistical analysis. All spectra (n = 21) in each category were initially reduced to continuous integral segments (bins) of equal width (0.04 ppm, each). The data obtained were normalised by dividing each integral segment by the total area of the spectrum in order to compensate for the differences in overall metabolite concentration between individual extracts. The spectral region between 0.51 and 7.89 ppm was binned and the data matrix was obtained. The regions 6.29–5.56, 5.02–4.21 and 3.38–3.31 were excluded from the analysis because of residual methanol signal and regions without signals in the methanolic extract. The final data-matrix obtained was subjected to Pareto scaling prior to principal component analysis (PCA), giving two orthogonal PC1 vs PC2 score plots. The grouping for the final PC1 vs PC2 score plot was obtained based on the metabolites such as citrate, caffeic acid, withanolides, withanamides and unassigned resonances between 4.20 and 3.39 ppm, which mainly comprise sugar signals as observed in the HSQC spectrum.

Results and Discussion

Fruit growth and development

Fresh weight, fruit area, moisture content, hexane soluble (oil) and aqueous methanolic soluble content in extracts of different development stages of W. somnifera fruits were examined (Fig. 1). There was a gradual increase in fruit weight and size as the fruits matured (Fig. 1A, B). Fresh weight of fruit ranged from 16.2 to 168.2 mg during the 35 days (stage I–VII) of fruit development. Fruit area increased from stage I (0.33 cm2 per fruit) to stage V (1.50 cm2 per fruit). However, there was a slight shrinkage in the fruits at maturity. Percentage hexane soluble (oil content) content ranged from 1.5 ± 0.2% (at stage I) to 14.3 ± 0.9% (at stage VII) w/w (Fig. 1B), whereas, a reverse trend was observed for the aqueous methanol soluble content from stage I to VII (15.7 ± 0.9 to 3.8 ± 0.08%). Similar trends in oil...
accumulation have been reported in developing almond seeds (Soler et al., 1988) and olive fruits (Nergiz and Engez, 2000).

Identification of metabolites
The metabolic shift during fruit maturation was investigated using $^1$H NMR-based metabolite profiling of the hexane and aqueous methanolic extracts. Metabolites from non-polar and polar extracts (Table 1) were assigned by combined use of 1-D and 2-D NMR spectra using standard methods (Annarao et al., 2008; Jayaprakasam et al., 2004; Matsuda et al., 2001; Sobolev et al., 2005).

Quantitative analysis of non-polar extracts
The quantification of metabolites in the hexane extract (in triplicate) was carried out by integration of specific NMR signals (Fig. 2). An integral value of each signal was taken for the calculation of percentage ratio of fatty acids present in the hexane extracts. Signals of the $\alpha$-methylene protons in the chain were used as a reference (Annarao et al., 2008). Integral values of $sn_1$ and $sn_3$ of triacylglycerol (TAG) at 4.30 ppm was used for the estimation of TAG, while the integral values of the resonance signals at 3.66 ppm ($s$, $-OCH_2$), 4.2 ppm ($t$,$-OCH_2CH_3$) and 4.08 ppm ($t$, $-OCH_2CH_2CH_2CH_3$) were used for the estimation of fatty acid methyl ester (FAME), ethyl ester fatty acid (EtFA) and butyl ester of fatty acid (BuFA), respectively. The free fatty acid (FFA) values were extrapolated using percentage contribution of TAG, FAME, EtFA and BuFA.

$^1$H NMR spectra revealed presence of small quantities of EtFA and BuFA in early developmental stages I and II (Table 2). FAME had a concentration of 0.86 and 0.43% at early developmental stages (I–II). It had decreased several-fold (0.13–0.21%) at stages III and IV. FFA accumulation showed a trend reverse to that of TAG. It was highest at stage I (45.3%) followed by a sharp decline until stage VI of fruit development (Table 2). Synthesis of TAG was low (51%) at stage I; however, it increased to 96% by the time fruits matured to stage VI. Thereafter, it declined slightly at the maturity stage VII, suggesting that stage VI is the time for harvesting *W. somnifera* fruits for maximum oil content. Similar
Table 1. $^1$H chemical shift assignments of the lipids in CDCl$_3$ along with $^1$H and $^{13}$C chemical shift assignments of polar metabolites in 4:1 binary mixture of CD$_3$OD and D$_2$O

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift (ppm)</th>
<th>13C (reference CH$_3$OH at 49.86 ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^1$H (reference CHCl$_3$ at 7.26/ CH$_3$OH at 3.34 ppm)</td>
<td></td>
</tr>
<tr>
<td>1. Sterols</td>
<td>0.68</td>
<td>Not detected</td>
</tr>
<tr>
<td>2. All fatty acids</td>
<td>0.89, 0.98, 1.20–1.40, 1.58, 2.05,</td>
<td>39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5,</td>
</tr>
<tr>
<td></td>
<td>2.29, 2.77, 5.35</td>
<td>24.5, 22.5, 20.8, 14.5, 128.0, 130.0, 179.0.</td>
</tr>
<tr>
<td>3. Methyl ester</td>
<td>3.66</td>
<td>Not detected</td>
</tr>
<tr>
<td>4. Ethyl ester</td>
<td>1.30, 4.22</td>
<td>Not detected</td>
</tr>
<tr>
<td>5. Butyl ester</td>
<td>1.35, 1.60, 4.05</td>
<td>Not detected</td>
</tr>
<tr>
<td>6. Triacylglycerol (TAG)</td>
<td>4.29–4.14, 5.25</td>
<td>Not detected</td>
</tr>
<tr>
<td>7. Alanine</td>
<td>1.51, 3.68</td>
<td>17.9, 53.0</td>
</tr>
<tr>
<td>8. GABA</td>
<td>1.94, 2.38, 3.03</td>
<td>25.4, 36.1, 41.2</td>
</tr>
<tr>
<td>9. Citrate</td>
<td>2.78–2.82</td>
<td>46.1</td>
</tr>
<tr>
<td>10. Aspartate</td>
<td>2.80, 2.98, 3.90</td>
<td>36.2, 53.1</td>
</tr>
<tr>
<td>11. Choline</td>
<td>3.21</td>
<td>55.4, 75.3</td>
</tr>
<tr>
<td>12. Phosphocholine</td>
<td>3.22</td>
<td>55.0, 75.5</td>
</tr>
<tr>
<td>13. Glucose</td>
<td>3.18, 3.48, 3.75, 3.83, 4.56, 5.17</td>
<td>63.2, 72.9, 74.1, 76.5, 78.1, 94.5, 98.7</td>
</tr>
<tr>
<td>14. Sucrose</td>
<td>3.46, 3.5, 3.81, 3.88, 4.04, 4.16, 5.42</td>
<td>62.4, 71.1, 76.2, 79.4, 85.2, 94.2</td>
</tr>
<tr>
<td>15. Withanolides</td>
<td>0.76, 0.81, 1.04, 1.15, 1.16, 1.87, 5.59–5.68</td>
<td>12.8, 13.0, 13.2, 14.9, 17.2, 126.4</td>
</tr>
<tr>
<td>16. Caffeic acid</td>
<td>6.45, 6.81, 6.95, 7.08, 7.42</td>
<td>115.7, 117.4, 119.0, 123.3, 143.1</td>
</tr>
<tr>
<td>17. Withanamides</td>
<td>2.89, 3.48, 6.72, 6.99, 7.08, 7.25</td>
<td>26.4, 41.7, 104.1, 112.9, 113.4, 122.8</td>
</tr>
</tbody>
</table>

Bold indicates the chemical shift of the proton used for quantification of metabolites.

Figure 2. Portions of $^1$H NMR spectra of lipid and sterol profile in the seven developmental stages of W. somnifera fruits.
Table 2. Percentage fatty acid composition at different fruit developmental stages of W. somnifera

<table>
<thead>
<tr>
<th>Fatty acid composition</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Stage V</th>
<th>Stage VI</th>
<th>Stage VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA</td>
<td>45.3 ± 0.3</td>
<td>17.7 ± 0.1</td>
<td>8.8 ± 0.6</td>
<td>7.9 ± 0.6</td>
<td>4.9 ± 0.7</td>
<td>3.6 ± 0.5</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>TAG</td>
<td>51.0 ± 1.6</td>
<td>80.7 ± 2.2</td>
<td>91.1 ± 2.7</td>
<td>91.9 ± 3.2</td>
<td>95.0 ± 3.6</td>
<td>96.3 ± 2.6</td>
<td>93.7 ± 2.8</td>
</tr>
<tr>
<td>FAME</td>
<td>0.86 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EtFA</td>
<td>0.9 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BuFA</td>
<td>1.9 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SFA</td>
<td>33.3 ± 1.3</td>
<td>23.0 ± 1.2</td>
<td>19.1 ± 1.1</td>
<td>18.2 ± 1.04</td>
<td>18.0 ± 1.0</td>
<td>18.5 ± 1.03</td>
<td>20.3 ± 1.6</td>
</tr>
<tr>
<td>MUFA</td>
<td>25.2 ± 1.5</td>
<td>18.5 ± 1.4</td>
<td>15.7 ± 1.1</td>
<td>18.6 ± 1.7</td>
<td>15.5 ± 0.9</td>
<td>24.9 ± 2.2</td>
<td>29.7 ± 1.8</td>
</tr>
</tbody>
</table>

(PEFA) C:18:2 37.1 ± 1.2 57.0 ± 1.5 64.6 ± 2.0 62.2 ± 2.0 65.7 ± 2.0 54.8 ± 1.8 48.5 ± 1.8
C:18:3 44.6 ± 0.6 1.4 ± 0.4 0.5 ± 0.04 1.1 ± 0.4 0.8 ± 0.08 1.8 ± 0.3 1.4 ± 0.3
USFA 66.7 ± 2.1 76.9 ± 2.5 80.8 ± 2.3 81.8 ± 2.2 82.0 ± 2.7 81.5 ± 1.9 79.7 ± 1.6
Sterols 11.0 ± 0.8 3.0 ± 0.4 1.1 ± 0.1 0.8 ± 0.09 0.9 ± 0.1 1.3 ± 0.11 1.2 ± 0.1

FFA, free fatty acids; TAG, triacylglycerol; FAME, fatty acid methyl ester; EtFA, fatty acid ethyl ester; BuFA, fatty acid butyl ester; SFA, saturated fatty acids; MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; USFA, unsaturated fatty acids; ±, standard error; ND, not detectable.

Metabolic analysis of polar extracts

NMR spectra of methanol–water extracts of all the seven fruit developmental stages of W. somnifera were extensively analysed by the combined use of one- and two-dimensional NMR experiments. The 1H, COSY and 1H-13C HSQC NMR spectra are shown in Figs. 3–8. The assignments were further confirmed based on the existing literature values (Jayaprakasam et al., 2004; Matsuda et al., 2001; Sobolev et al., 2005). The stack-plot of 1H NMR spectra of fruit development stages showed signals of alanine, aspartate, citrate, choline, phosphocholine and GABA in the high-field region (Fig. 3). Signals from diverse sugars included free and bound glucose (Gluc), sucrose (Sucr) and sugar units in the mid-field region (Fig. 4), and signals from caffeic acid, withanolides and withanamides in the down-field region (Fig. 4).

Identified polar metabolites were quantified by integrating the distinct signals of each metabolite with respect to the intensity of the nine protons of TSP (in D2O, 0.375% w/v) on the dry weight basis of fruits using the following equation (Diehl et al., 2007):

Weight of metabolite = \( \frac{Mw\ metabolite}{Mw\ TSP} \times \frac{No.\ of\ HTSP}{No.\ of\ H\ metabolite} \times \frac{Integral\ area\ of\ metabolite}{Integral\ area\ of\ TSP} \times wt\ of\ TSP \)

The quantitative variability in metabolites among seven developmental stages of W. somnifera fruits is presented in Fig. 9(A–F). Alanine, aspartate, choline and phosphocholine were detected in all the seven stages of fruit development. Alanine, choline and aspartate concentration decreased from stage I as the fruit development progressed. Alanine decreased from 5.2 ± 0.76 to 1.1 ± 0.06 mg/g at stage V. Similarly, choline fell from 5.10 ± 0.58 to 0.97 ± 0.03 mg/g in mature fruits. Phosphocholine concentration was nearly one-sixth to that at stage I (1.83 ± 0.15 mg/g). Aspartate was detected at 19.4 ± 1.05 mg/g at stage I but declined substantially to 1.6 ± 0.18 mg/g as the fruits matured. Stage II of fruit development showed presence of γ-amino butyric acid (GABA); however, its concentration gradually decreased and eventually decreased near maturity. Akhiro et al. (2008) reported high concentration of GABA in tomatoes before the breaker stage; however, it rapidly catabolised at later stages.

Caffeic acid (3,4-dihydroxycinnamic acid), one of the most common phenolic acids of fruits, was a major metabolite at the initial stage (130 ± 3.77 mg/g) but declined sharply at subsequent stages and was not detected in mature fruits. This was in contrast to the observation made by Arancibia-Avila et al. (2008), who reported significantly higher content of caffeic acid in ripe fruits of durian (Durio zibethinus). The resonance arising due to citrate was first observed at stage IV and increased sharply until maturity of the fruits. Similar increase in the levels of citrate and malate has been reported in developing fruits of strawberry (Iannetta et al., 2004).

The highest concentration of sucrose (56.7 ± 1.78 mg/g) was observed at stage I. It declined in the middle stages but had an upsurge at maturity. Signals of glucose (anomeric proton) were first observed at stage II (18.7 ± 1.9 mg/g). Its concentration observations have been made by Wiberg and Bafor (1995) in developing palm (Elaeis guineensis) kernels, Mazhar et al. (1998) in sunflower, and Annarao et al. (2008) in Jatropha curcas.

Percentage composition of FFA, saturated fatty acid (SFA), unsaturated fatty acid (USFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and sterols at different stages of fruit development has been given in Table 2. There were significant variations in percentage composition of various fatty acids at different stages of development. No EtFA and BuFA were observed except small percentages at very early stage of fruit development. Percentage composition of TAG increased nearly 2-fold from early stage to maturity. It was also interesting to observe that FFA composition decreased substantially as the fruits matured. Linoleic acid composition increased nearly 2-fold from early stage to middle stage. It remained nearly constant in the following stages. A similar observation has been recorded during ripening of coriander (Coriandrum sativum) fruits by Msaada et al. (2009).
Figure 3. The expanded spectra of high-field region (polar extract) in the seven fruit development stages of *W. somnifera*.

Figure 4. The expanded spectra of low-field region (polar extract) in the seven fruit development stages of *W. somnifera*. 
Figure 5. Phase-sensitive DQF-COSY spectrum from two different regions (0.5–6.0 and 5.0–7.9 ppm) of stage I showing Ala (alanine), Asp (aspartate), Cfa (caffeic acid), GABA (γ-amino butyric acid), Gluc (glucose), Sucr (sucrose) and Wl (withanolide).

Figure 6. HSQC spectrum of stage I of *W. somnifera* fruit.
Figure 7. Phase-sensitive DQF-COSY spectrum from two different regions (0.5–5.65 and 5.3–7.5 ppm) of stage VII showing Ala (alanine), Asp (aspartate), Citr (citrate), GABA (γ-amino butyric acid), Gluc (glucose), Sucr (sucrose) and Wm (withanamide).

Figure 8. HSQC spectrum of stage VII of *W. somnifera* fruit.
followed a trend similar to that of sucrose. This was in conformity with earlier report of McCollum et al. (1988) who reported a similar trend in sucrose at early stage of fruit development in muskmelon.

Concentration of withanolides was highest (16.6 ± 1.17 to 27.7 ± 1.19 mg/g) at initial stages; however, it decreased sharply in the middle stages and had relatively lower concentration (3.1 ± 0.67 to 4.1 ± 0.75 mg/g) at maturation stages, while signals of withanamides were first observed at stage III (4.6 ± 0.89 mg/g) and increased significantly (17.2 ± 1.15 mg/g) until the matured stage (stage VII). Withanolides have been reported to be beneficial in the treatment of tumours and inflammation (Jayaprakasam et al., 2003), whereas, withanamides have antioxidant properties and also help in the prevention and progression of Alzheimer’s disease (Jayaprakasam et al., 2004, 2009). The present study assumes significance since mature fruits have insignificant quantities of withanolides as compared to very young fruits. However, withanamides have been detected in relatively substantial quantity in matured fruits. These findings may be useful for developing herbal dietary supplements for nutraceutical industry.

The PCA1 vs PCA2 score plot along with its longitudinal loading and percentage variation of each PC of polar metabolites from seven fruit development stages is shown in Fig. 10(A–C). The clusters observed in the score plot can grossly be classified into three metabolic phases, viz. initial phase (stages I and II); development phase (stages III–V); and maturation phase (stages VI and VII). The initial phase had relatively higher concentrations of alanine, aspartate, choline, phosphocholine, sucrose, caffeic acid and withanolides. This suggested high metabolic activity during the initial stage of fruit development. The developmental phase was marked by the presence of citrate, which was not observed in the initial phase. This phase had relatively low concentrations of alanine, aspartate, choline, GABA, caffeic acid, glucose, sucrose and withanolides. The maturation phase showed a higher accumulation of citrate and withanamides. The decrease in caffeic acid content at maturity could be due to its metabolic re-routing towards biosynthesis of anthocyanin as fruits turned yellow-pinkish from green at this stage. Similar increase in purple colouration and the levels of anthocyanin have been reported in developing fruits of tomato (Mathews et al., 2003).

Figure 9. Quantitative variability of primary and secondary metabolites in the seven developmental stages of *W. somnifera* fruits. Error bar represents standard error.
Figure 10. Score plot of PC1 vs PC2 (A); loading plot (B); and principal components accounts the cumulative percentage variation (C) of polar metabolites at the seven fruit developmental stages of *W. somnifera*. 
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