# Research Journal of
# Phytochemistry

ISSN 1819-3471

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Phytochemistry 8 (2): 25-34, 2014
ISSN 1819-3471 / DOI: 10.3923/rjphyto.2014.25.34


# *In vivo* and *in vitro* Variation in Protein Profiling in *Withania somnifera* (L.) Dunal

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## ABSTRACT

Proteomics is a leading new technology for the high-throughput analysis of proteins on a genome-wide scale in living system. This technique was applied to investigate the protein changes under *in vitro* and *in vivo* conditions, since *in vitro* cultures is considered to be an alternative approach to traditional agriculture in the industrial production of the biomolecules. The overall goal of this project was to investigate the changes in protein expression under *in vitro* and *in vivo* leaves tissues of *Withania somnifera*. Of ~28 protein bands resolved in the two-dimensional gels, 21 protein bands were similarly expressed in both *in vitro* and *in vivo* root tissues 1 protein in *in vivo* condition and 2 protein bands were differentially expressed only in *in vitro* tissue. This is the first report on the comparison of *in vitro* and *in vivo* samples by establishment of a 2-D reference proteome map of *Withania somnifera*.

**Key words:** *Withania somnifera*, ashwagandha, proteome, two dimensional electrophoresis

## INTRODUCTION

*Withania somnifera* Dunal (Solanaceae), known in India as ashwagandha or winter cherry, is one of the most valuable plants of the traditional Indian systems of medicines, is used in more than 100 formulations of Ayurveda, Unani and Sidha and is therapeutically equivalent to ginseng (Sangwan *et al*., 2004). Its ginseng like health-promoting effects has earned it the popular name of Indian ginseng. The biologically active chemical constituents are steroidal compounds, including ergostane type steroidal lactones, withaferine A, withanolides A-Y, withasomniferin-A, withanone, etc. (Ganzera *et al*., 2003) alkaloids (ashwagandhine, cuswhygrine, anahygrine, tropine, etc.). Many pharmacological studies have been carried out to describe multiple biological properties of *W. somnifera* (Mishra *et al*., 2000).

Different plant parts of various species of the genus *W. somnifera* have exhibited varied pharmacological activities, such as anti-inflammatory hypocholesterolemic (Asthana and Raina, 1989), cardiac and cerebral ischemia (Keller *et al*., 1998), immunomodulator and antitumor effect (Agarwal *et al*., 1999), neurodegenerative disorders (Perry *et al*., 2000), carcinogenesis (Kamat and Devasagayam, 2000), rheumatic disorders (Hanninen *et al*., 2000), anticancer and arthritis (Prakash *et al*., 2001), contributes a major role in the ageing process (Khodr and Khalil, 2001), anxiety and anti-depression (Al-Hindawi *et al*., 1989; Archana and Namasivayam, 1999; Bhattacharya *et al*., 2001; Singh *et al*., 2001), antioxidants (Ferguson, 2001; Tang *et al*., 2001), diabetes (Gorogawa *et al*., 2002), chronic stress (Archana and Namasivayam, 1999;

Dhuley, 2000; Kaur *et al*., 2001; Bhattacharya and Muruganandam, 2003), anti-cancer chemotherapy and radio-sensitization (Devi *et al*., 1995; Devi, 1996; Chang *et al*., 2007; Ojha and Arya, 2009). As leaves contain a number of therapeutically applicable withanolides, mass cultivation of leaves *in vitro* will be an effective technique for the large scale production of these secondary metabolites.

Protein Profiling, an independent, emerging sub-specialty of proteomics, is poised to provide unprecedented insight into biological events. Protein profiling is defined here as the quantitative assessment of protein expression levels. As profiling evolves, the term will increasingly refer to the study of multiple proteins, protein forms or protein families, almost always comparing two different states (De Palma, 2006). Protein profiling in the high throughout mode is a most useful technique that allows formation of reference databases for cells and tissues and performance of comparative proteomics. The analysis of all proteins (proteome) and all metabolites (metabolome), however, continued to pose significant challenges. Proteins and metabolites are more divert and biochemically heterogenous, which precludes the application of a single standardized procedure for their analysis (Bino *et al*., 2004). In recent years SDS-proteins has been found wide application in resolving genetic diversity and for intra and interspecific studies.

These technologies will provide novel methods for early detection and diagnosis of cancer as well as classification and prognostic prediction. Proteomic profiling will also lead to better targeted therapies to enable the delivery of personalized medicine. Proteomic methods thus have the advantage of identifying the dynamic and transient interactions that are the sum of all molecular interactions impinging on a particular cellular pathway at the moment of analysis. Proteomics offers great potential for studying mechanisms of post translation regulation as well as biosynthetic pathways. However it has not been widely applied in plant biology. Currently, the complete genome of a number of plant species has been sequenced (Tabata, 2002; Frazier *et al*., 2003; Kav *et al*., 2007). However the functions of many of the identified genes remain unclear. Thus it is important to shift the focus towards the functional characterization of proteins that are encoded by the cellular genetic machinery (Kav *et al*., 2007; Senthil *et al*., 2011).

Global analysis of the system components (DNA, RNA, proteins and metabolites) is now possible, although, at different analytical depth at present. Protein profile system can be used as biochemical marker for selection superior genotype of the medicinally important plant. The protein banding profile system revealed the biochemical variation and evolutionary relationship. Protein profiling in high throughout mode is relatively simple and provides a snapshot of the major protein constituents of the cell (Yates, 2004). Each allele codes for the production of amino acids that string together to form protein. Thus differences in the nucleotide sequence of allele result in the production of slightly different strings of amino acids or variant forms of the proteins. These protein codes for the development of the anatomical and physiological characteristic of the organism, which are responsible for determining aspects of the behaviour of the organism (Shibata, 2005).With the advent of proteomics and mass spectrometry, systematic identification of proteins has become possible, as demonstrated in several studies in different organisms (Tyers and Mann, 2003). Proteins are major actors involved in many physiological processes, so profiling is used to predict those processes (Voelckel *et al*., 2010). Protein profiling of many species has been reviewed by few workers (Kumar and Kumari, 2009; Johnson, 2010; Shim *et al*., 2010; Hew and Gam, 2010). However the work on the protein profiling of *Withania* Species has been done by (Senthil *et al*., 2011). But no systematic efforts have been made to study protein expression of *W. somnifera*.

The present study was designed to investigate the protein changes under *in vitro* and *in vivo* conditions. Separation of proteins in *in vitro* and *in vivo* tissues of *W. somnifera* was done using 2-D gels.

## MATERIALS AND METHODS

### Proteome analysis

**Sample preparation:** The 21 to 28 days old calli was harvested for analysis kept at above 90°C for 3 to 5 min in a hot air oven to inactivate enzyme activity followed by continuous drying at 50°C to 60°C for 6 to 72 h (Jain *et al.,* 2004). Dried callus was homogenized to fine powder and further exploited for protein extraction. Dried whole plant and powdered it with an electric homogenizer.

**Protein extraction:** The dried calli and dried plant were submitted to extraction with protein extraction buffer (Table 1).

The samples were sonicated and clarified by centrifugation at 1500 rpm for 10 min. The clear supernatants were collected and kept in boiling water for denaturation. These protein samples were stored at -20°C for long time. Supernatants were used as a sample for polyacrylamide gel electrophoresis.

**Protein quantification:** The protein content in the different fractions was performed according to Bradford (1976). A stock solution of Bovine Serum Albumin (BSA; Sigma Chemical Co., St. Louis, USA) was prepared (1 mg $mL^{-1}$), out of which 0.2-1.0 mL of the standard was taken in separate test tube and volume in each case was raised to 2 mL by adding double distilled water. To each, 3 mL of Bradford reagent was added, mixed and kept at 37°C for 10 min and absorbance was measured at 595 nm. Similarly, 20 μL of extracted protein was diluted to 25 times in TBE buffer and in 0.5 mL of sample 1.5 mL of double distilled $H_2O$ and 3 mL of Bradford reagent was added to and the mixture was allowed to cool and absorbance was measured at 595 nm. Graph was drawn between concentration of standard protein and absorbance. The quantities of unknown samples were measured on the basis of standard curve and dilution factor.

**Sample preparation for SDS-PAGE:** Electrophoresis of equal volume of protein extracted from equal quantity (0.5 g) of sample was carried out on 12% polyacryamide gel. Since amount of sample and buffer were same in all samples, it was assumed that quantity of protein in mg $mL^{-1}$ is as per expression level of proteins. In order to load protein samples properly for SDS-PAGE the extracted protein samples were mixed with 10 X bromophenol blue dye. The sample so prepared contained, 34 μL of extracted protein as per procedure described earlier and 2 μL 10X Bromophenol blue dye.

**Preparation of polyacrylamide gel (Sambrook *et al.,* 1989):** In order to resolve various polypeptides SDS-PAGE was conducted using 12% resolving gels. A 12% separating gel (25 mL) was used for resolving the polypeptides whereas 5% stacking gel (5 mL) was used to stack the polypeptides. Glass plates and spacers were properly cleaned with double distilled water followed by spirit. Plates are set in the gel caster and sealed with the help of sealing agar. Whole cassette of glass plate and spacers were assembled properly. Vaseline or tape was used at bottom of cassette to prevent leakage of gel solution. Separating gel solution for 12% gel (Table 2) was prepared in conical flask and degassed using vacuum pump for 4-5 min until bubbles stopped forming at the surface.

Separating gel solution was then poured in the chamber between the glass plates leaving the space for stacking gel. Distilled water was overlaid to form about a 2-5 mm layer and left to set for 30 min. On polymerization of the separating gel, water overlay was carefully removed by inverting

Table 1: Composition of protein extraction buffer

| | |
|---|---|
| 0.5 M Tris-HCl (mL) | 2.5 |
| Urea (g) | 9.6 |
| SDS (g) | 1.0 |
| Glycerol (mL) | 4.0 |
| BME (µL) | 500 |

Table 2: Solution components for resolving and stacking gel

| Gel component | 12% 25 mL resolving gel | 5% 5 mL stacking gel |
|---|---|---|
| Dd $H_2O$ | 8.2 | 3.4 |
| 30% polyacrylamide (29:1) | 10.0 | 0.83 |
| 1.5 M Tris-HCl pH 8.8 | 6.3 | - |
| 1 M Tris- HCl pH 6.8 | - | 0.63 |
| 10% SDS | 0.25 | 0.05 |
| 10% APS | 0.25 | 0.05 |
| TEMED | 0.01 | 0.005 |

the whole cassette. Immediately after overlaying water from cassette, APS and TEMED was in stacking gel and poured on the surface of the separating gel. The teflon comb was then placed in stacking gel. The comb was removed carefully after 10 min and washed the wells with dd $H_2O$ to remove air bubbles. The gel was allowed to polymerize for at least 2 h at cool temperature. The gel assembly was removed from the casting stand and snapped it into the cooling core. Cooling core with clamped gel plates and one dummy plate was placed in vertical electrophoresis tank. Prepared samples were then loaded in the wells with protein molecular weight marker at one end. Upper and lower tanks were then filled with Tris-glycine buffer (1X) to complete the circuit. The power supply was turned to 100 V till the samples were in stacking gel then turned to 200 V until the bromophenol blue reached the bottom of the resolving gel. After completion the power supply was turned off and the cooling core unit was pulled out from electrophoresis tank, the unit was disassembled and gel was detached from glass plates. The gel was placed in coomassie brilliant blue stain for overnight. After complete staining, gel was destained in destaining solution on a slowly rocking platform and changed the destaining solution 3-4 times, till the background became quite clear. After destaining the gel was analysed on densitometer (Bio RAD Multi-Analyst). The multi-analyst software of densitometer gave quantity and intensity of each band of every lane. The gel was scanned and photographed.

The identification/comparison of the components of the storage proteins in the extract was made using a protein marker (Banglore Genei). The molecular weights of the marker corresponded to 20.1, 29, 43, 66 and 97.4 kDa.

## RESULTS AND DISCUSSION

Two-dimensional polyacrylamide gel electrophoresis (2-D) is an established and powerful technique for analyzing the complex mixtures of protein. Proteomic analysis offers a new approach to identify a broad spectrum of genes that are expressed in living system. We applied this technique to investigate the protein changes under *in vivo* and *in vitro* conditions, since *in vitro* cultures is considered to be an alternative approach to traditional agriculture in the industrial production of the biomolecules. To better understand the proteins and enzymes involved in biosynthetic pathway, detailed two-dimensional gel electrophoresis (2-D) of *in vivo* leaves samples and *in vitro* grown

callus of *W. somnifera* were conducted. Total soluble proteins were extracted and then total amount of proteins were estimated using Bradford method. The concentration of proteins in estimated sample was found to be from 3.8 to 7.9 mg 120 $mg^{-1}$). Finally the equal amount of extracted and estimated proteins subjected to SDS-PAGE to generate banding pattern with a protein molecular weight marker (PMW-M from G Brand). After electrophoretic separation of proteins, 28 band positions were observed and finally scored for analysis. 23 protein spots were similarly expressed in both *in vivo* and *in vitro* tissues 1 protein in *in vivo* condition and 2 protein spots were differentially expressed only in *in vitro* tissue. Total proteins were analyzed by Bradford assay method. Unlike Lowry's method, metal ions such as $NH_4^+$, $Na^+$, $K^+$ phenols and carbohydrates such as sucrose do not interfere in this assay(Kumar *et al.*,2010). The standard curve was prepared on the basis of absorbance at 595nm from the standard curve, the concentration of protein was found to be 6.8 and 4.3 mg $g^{-1}$ in *in vivo* plant and *in vitro* (callus) (Table 3, 4). The report of Cormack *et al.* (2001) suggested that leaves record high amount of proteins compared to roots and seeds. Generally, 18% proteins were profiled in leaves whereas roots have less than 0.25% protein in *Arabidopsis thaliana* root. The quantity of the protein content in the leaves of *W. somnifera* was analyzed and it was observed that leaves contain high amount of protein compared to roots and seeds (Khanna *et al.*, 2006).

Comparative analysis of 2-D gels revealed high level of similarity in the protein pattern of both *in vivo* and *in vitro* leaves sample. The 23 spots were commonly present in the *in vivo* and *in vitro* leaves samples. One for presence and zero for absence was considered and tabulated in electrophoretic banding pattern. Twenty three bands with molecular weights 99.2, 96.4, 94.1, 92.6, 88.2, 64.2, 60.2, 55.2, 52.3, 50.2, 48.8, 40.2, 38.2, 37.1, 36.9, 36.2, 34.2, 29.5, 28.1, 27.2, 26.2, 22.3 and 19.2 kDa were expressed in both conditions (Fig. 1; Table 5). The intensity of bands was high in *in vivo* sample as compared to *in vitro* sample (Table 6).

Interestingly, two (*in vitro* specific protein) differential spots were present in the *in vitro* (66.2 and 39.6 kDa) that were not found in the *in vivo* leaves. These proteins accumulated in immature cells grown *in vitro* conditions that permits callus formations. These may be callus associated protein for proliferative growth and cellular differentiation (Table 5).

The protein profiles indicate that most of the proteins present in leaves and callus was of a same molecular weight. But in callus protein bands of (39.6 kDa) molecular weight region were not detected. The protein bands of molecular weight indicate that the number of protein identified in

Table 3: Observation table for known protein solution

| Standard solution (mL) | d $H_20$ (mL) | Bradford reagent (mL) | Absorbance at 595 nm |
|---|---|---|---|
| 0 (Blank solution) | 2 | 3 | -- |
| 0.2 | 1.8 | 3 | 0.285 |
| 0.4 | 1.6 | 3 | 0.487 |
| 0.6 | 1.4 | 3 | 0.65 |
| 0.8 | 1.2 | 3 | 0.724 |
| 1.0 | 1.0 | 3 | 0.822 |

Table 4: Observation table for unknown leaf and callus protein solution

| S. No. | Test solution (mL) | d $H_20$ (mL) | Bradford reagent (mL) | Absorbance at 595 nm | Protein (mg $g^{-1}$) |
|---|---|---|---|---|---|
| *In vivo* | 0.5 | 1.5 | 3 | 0.518 | 6.8 |
| *In vitro* | 0.5 | 1.5 | 3 | 0.323 | 4.3 |

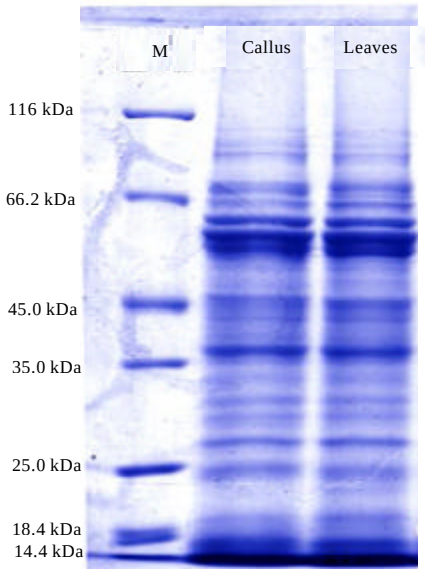


Fig. 1: *In vivo* and *in vitro* protein profiling of isolated protein from *Withania somnifera* L.

Table 5: Electrophoretic banding pattern of *in vitro* and *in vivo* samples derived from SDS-PAGE of leaf and callus proteins

| Band | Molecular weight (kDa) | Plant leaves (*in vivo*) | Callus (*in vitro*) |
|---|---|---|---|
| 1 | 99.2 | 1 | 1 |
| 2 | 96.4 | 1 | 1 |
| 3 | 94.1 | 1 | 1 |
| 4 | 92.6 | 1 | 1 |
| 5 | 88.2 | 1 | 1 |
| 6 | 66.2 | 0 | 1 |
| 7 | 64.2 | 1 | 1 |
| 8 | 60.2 | 1 | 1 |
| 9 | 55.2 | 1 | 1 |
| 10 | 52.3 | 1 | 1 |
| 11 | 50.2 | 1 | 1 |
| 12 | 48.8 | 1 | 1 |
| 13 | 40.2 | 1 | 1 |
| 14 | 39.6 | 0 | 1 |
| 15 | 39.2 | 1 | 0 |
| 16 | 38.2 | 1 | 1 |
| 17 | 37.1 | 1 | 1 |
| 18 | 36.9 | 1 | 1 |
| 19 | 36.2 | 1 | 1 |
| 20 | 34.2 | 1 | 1 |
| 21 | 29.5 | 1 | 1 |
| 22 | 28.1 | 1 | 1 |
| 23 | 27.2 | 1 | 1 |
| 24 | 26.2 | 1 | 1 |
| 25 | 22.3 | 1 | 1 |
| 26 | 19.2 | 1 | 1 |
| 27 | 14.4 | 1 | 1 |
| 28 | 12.1 | 1 | 1 |

1: Presence, 0: Absence

Table 6: Electrophoretic densitogram of *in vitro* and *in vivo* samples of leaf and callus proteins

| Band | Molecular weght (kDa) | Plant leaves (*in vivo*) | Callus protein (*in vitro*) |
|---|---|---|---|
| 1 | 99.2 | ++ | + |
| 2 | 97.4 | +++ | 0 |
| 3 | 94.1 | ++ | 0 |
| 4 | 92.6 | + | 0 |
| 5 | 88.2 | + | 0 |
| 6 | 64.2 | + | 0 |
| 7 | 61.2 | + | 0 |
| 8 | 60.2 | + | 0 |
| 9 | 48.8 | ++ | 0 |
| 10 | 46.2 | 0 | 0 |
| 11 | 45.5 | +++ | 0 |
| 12 | 44.2 | 0 | +++ |
| 13 | 43 | +++ | + |
| 14 | 42.3 | 0 | +++ |
| 15 | 41.9 | 0 | 0 |
| 16 | 40.5 | ++ | 0 |
| 17 | 39.2 | + | 0 |
| 18 | 37.1 | + | + |
| 19 | 32.2 | ++++ | +++ |
| 20 | 29.5 | ++++ | +++ |
| 21 | 28.1 | + | + |
| 22 | 26.2 | 0 | 0 |
| 23 | 26.2 | +++ | ++ |
| 24 | 22.3 | ++++ | +++ |
| 25 | 20.6 | ++++ | +++ |
| 26 | 19.2 | ++ | 0 |
| 27 | 14.4 | ++ | ++ |
| 28 | 12.1 | + | + |

*in vitro* condition was more compared to *in vivo* condition and for development of callus it required the same protein compared to *in vivo* condition.

## CONCLUSION

Until now no reports are available on the comparison of *in vivo* and *in vitro* leaves in plants of commercial importance. Hence pioneering attempt has been made to study the profile of proteins expressed in *in vivo* and *in vitro* samples of *W. somnifera*.

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