

Varietal Comparison of Withanolide Contents in Different Tissues of *Withania somnifera* (L.) Dunal (Ashwagandha)

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ABSTRACT

Five varieties of Ashwagandha (*Withania somnifera* L. (Dunal) i.e. Chetak, Pratap, Nimithli, Poshita and Jawahar-20) were analyzed for withanolide A and withaferin A. These components were evaluated from leaf, stem, root and seeds using HPLC. These components were present in all these tissues. However, their content varies from tissues to tissue and variety to variety viz (Chetak, Pratap, Nimithli, Poshita and Jawahar-20). Withaferin A and withanolide A contents were found to be highest in Poshita followed by Jawahar-20. Withaferin A and withanolide A contents were also evaluated and compared in field-grown and *in-vitro* grown plants of Poshita and Jawahar-20. Callus derived from Poshita root showed the highest withaferin A content however, withanolide A was found to be highest in callus derived from Jawahar-20 leaf. Thus this study indicated that promoting an elite variety through tissue culture with consistency in the withanolide contents can be a promising approach to meet the growing demand of Ashwagandha.

Key-words: *Withania somnifera*, Withaferin A, Withanolide A, HPLC, Field grown, *In-vitro* grown, Ashwagandha

INTRODUCTION

Withania somnifera (L.) Dunal, popularly known as Ashwagandha, is a high valued medicinal plant of India. It is a priority medicinal plant which is identified by National Medicinal Plant Board of India. The roots and leaves of Ashwagandha contain various alkaloids viz withanolides ^[1] and withaferin A ^[2]. Among withanolides, withaferin A and withanolide A have been reported to be dominant metabolite distributed among various tissues of this plant in varying concentrations ^[3].

Ashwagandha is conventionally propagated by seeds but the percentage of germination is low. The conventional propagation method cannot meet the increasing demand of this plant used as raw material for the preparation of pharmaceutical products. Tissue culture technique can be an alternative for the continuous production of plantlet stocks for large scale field cultivation and shoot multiplication. The Uttarakhand has identified seven districts as agri export zone for medicinal and aromatic plants looking towards the export potential of medicinal plant products. However, the major bottleneck for promoting these activities is its availability of quality planting material/elite germplasm for enhanced active ingredients. The withanolides and steroidal compounds bear resemblances, both in action and appearance, to the active ginsenosides of Asian Ginseng. The plant has been realized as a plant of great medicinal potential with wide spectrum applications of withanolides and alkaloids

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from root and leaf extract and it is regarded as Indian ginseng. Since environmental factors influence the secondary metabolite biosynthesis, therefore, it was important to assess the withanolide contents in elite germplasm and compared the withanolide contents in field grown and *in-vitro* grown promising varieties. Several chemotypes exist that differ in their withanolide content [4].

The estimated production of Ashwagandha roots in India is more than 1500 tonnes and the annual requirement is about 7000 tonnes [5]. For commercial withanolide production, field plant material has generally been used but the quality of these plant products may be highly affected by different environmental conditions.

In view of this, in the present study we have assessed the withanolides (withaferin A and withanolide A) contents in five different varieties of Ashwagandha grown in Pantnagar and investigated the distribution of withanolides contents in different tissues of the plant grown in *in-vitro* and field grown plant.

MATERIALS AND METHODS

Collection of plant material- Field grown Leaves, stem and roots of five different varieties of *Withania somnifera* viz. Jawahar-20, Poshita, Chetak, Nimithli and Pratap used for the experimentation were procured from Mango Garden, G. B Pant University of Agriculture & Technology, Pantnagar (Altitude 242 m, Latitude 293°.000"N, Longitude 7931°0.120"E). For *in-vitro* grown seedlings, seeds of the above mentioned five varieties were purchased from CIMAP, Lucknow, India. Field grown plants were 2 years old while *in-vitro* grown seedlings/ callus were 6 months old.

Fresh leaves, stems and roots were washed under tap water to remove soil and dust particles and were oven dried for 3 days at 40°C. Dried leaves and roots were then crushed with the help of pestle and mortar to make powder. Methanolic extracts were prepared from these powdered plant materials. Standards of Withanolide A and Withaferin A were purchased from Natural Remedies, Bangalore.

Sterilization of seeds for germination- Sterilization of seeds was done [14] with some modifications. The seeds of two promising varieties viz. Jawahar-20 and Poshita were washed under tap water. After surface sterilizing with tween-20 and then rinsed with Distilled water 5–6

times. Inside the laminar air flow hood seeds were further sterilized with 0.5% Bavistin and rinsed with distilled water 3 - 4 times. Seeds were further treated with 0.1% mercuric chloride and rinsed with distilled water 3 - 4 times. Then finally they were treated with 70% ethanol and rinsed with distilled water 3 - 4 times. Then the seeds were placed in half media without any growth regulators/hormones and incubated at 25°C under 16 h photoperiod.

For callus induction from leaf and root explants- Leaf and root explants were taken from 6 months old *in-vitro* grown seedlings of elite genotypes of Ashwagandha. The explants were placed in sterile petri-plates and were pricked with sterile needle 10-20 times and were placed on MS media [15] containing different hormone combinations 2,4-D (2 mg/l) and Kinetin (0.2 mg/l). Sealed jam bottles were incubated at 25°C under 16 h photoperiod.

Extraction and HPLC analysis- Extraction and HPLC analysis of withanolide A/ withaferin A were carried [16] with some modifications.

Preparation of Seeds, Leaves, stem and roots sample for HPLC- Fresh leaves and roots were washed under tap water to remove soil and dust particles and oven dried for 3 days at 40°C. The dried seeds, leaves, stem and roots samples were crushed with the help of pestle and mortar to make powder. The powdered material was weighed and percolated with 80% methanol and sonicated for 20 min. The methanolic extract was subjected to filtration by whatmann filter paper. These steps were repeated thrice. Then filtrate was pooled and evaporated on vacuum rotatory evaporator at 60°C. The dried residue was re-dissolved in 4ml methanol. A pinch of charcoal was added to the extract to decolorize the sample and centrifuged at 8000 rpm for 15 min. The supernatant was then filtered through nylon filter membranes (0.22 µ). The samples, thus prepared were kept in the vials at 4°C and ready for injection for future use.

Preparation of solvent- Methanol and water were mixed in the appropriate ratio (70:30) and filtered through nylon filter membranes (0.45 µ) with the help of vacuum pump. Then the solvent was subjected to water sonication for 10 minutes for removal of air bubbles.

Injection of Seeds, Leaves, stem and roots sample in HPLC equipment- The withanolide fractions were analyzed using HPLC (Agilent 1120 Compact LC) equipped with C18, 5 μ m (ODS34.6 \times 250 mm) column. The mobile phase was a mixture of methanol and water (70:30 v/v) at a flow rate of 1 ml min⁻¹ and column temperature was maintained at 30°C. The detection wavelength was set at 254 nm. The chromatography system was equilibrated by the mobile phase. 10 μ l of sample with the help of Hamilton syringe was injected into the injection port of HPLC Equipment. The sample was allowed to run for the pre-set run time. The retention time and peak area of the peak of interest were observed. With the help of standard curve, the quantification of withanolides in the samples was done.

Statistical Analysis- The statistical analysis was done using Two-way ANOVA and Bonferroni post test. The software used for the Graph pad prism analysis.

RESULTS AND DISCUSSION- The separation of methanolic extracts of different field grown varieties (Jawahar-20, Pratap, Nimithli, Poshita and Chetak); 2 years old) of *Withania somnifera* with respect to standards under optimized conditions is shown in Fig. 1 (a, b and c). From the HPLC profiles it was found that the total withaferin A contents was highest in Poshita genotype i.e. 4.675 mg/g (DW) followed by Jawahar-20 i.e. 1.986 mg/g (DW) and least was found in Pratap i.e. 0.21 mg/g (DW). While total withanolide A contents was found to be highest in Poshita i.e. 0.814 mg/g (DW) followed by Jawahar-20 i.e. 0.678 mg/g (DW) while least in Chetak i.e. 0.122 mg/g (DW) (Fig. 2: a & b).

The different parts of the *Withania somnifera* plant (field grown) viz. (Seed, Leaf, Root, and Stem) taken from 2 years old varieties were evaluated for withaferin A and withanolide A contents. It was observed that the withaferin A contents in seed ranged between 0.181 to 0.217 mg/g (DW), in leaves it ranged between 0.024 to 0.775 mg/g (DW), in roots it ranged between 0.002 to 0.821 mg/g (DW) while in stem it ranged between 0.003 to 1.873 mg/g (DW). Study of leaf, root, stem, flower and berry tissue from *Withania somnifera*-JA 20 varieties at five different life-cycles was done and samples were used for HPTLC, gene expression and RNA extraction. [3]

Analysis of withanolide contents in root, stem and leaf (*in-vitro* and greenhouse) confirmed the presence of

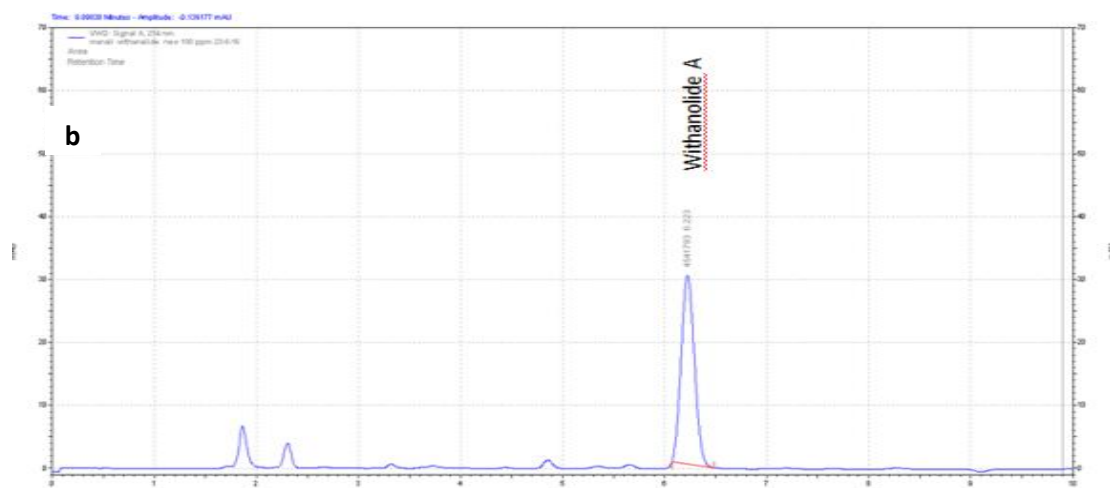
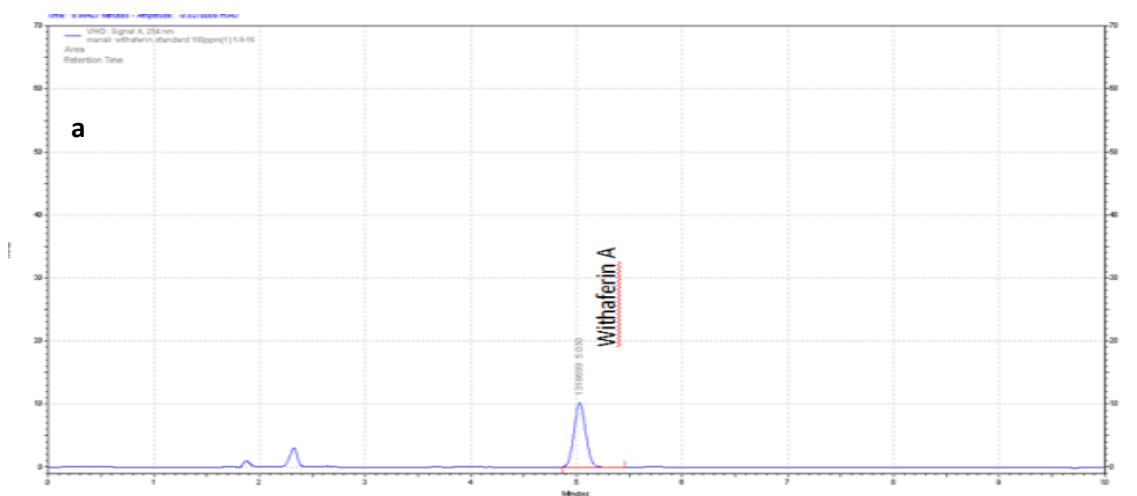
withaferin A and withanolide A in all the parts but significant differences in their ratio. Withaferin A was most dominant in leaves from both (*in-vitro* and greenhouse) while very minor in roots. Stem contained the lowest amount of total withanolide (withaferin A and withanolide A) [17]. This could be explained by different environmental conditions that may influence the synthesis of withanolide. It was also found that among the different explants of different varieties, the withaferin A content was highest in Poshita stem i.e. 1.873 mg/g (DW) followed by Jawahar-20 stem i.e. 1.471 mg/g (DW). While least withaferin A content was recorded in Pratap root i.e. 0.002 mg/g (DW). On the other hand withanolide A contents in seeds ranged between 0.046 to 0.081 mg/g (DW), in leaves between 0.015 to 0.044 mg/g (DW), in roots between 0.031 to 0.149 mg/g (DW) while in stem it ranged between 0.026 to 0.252 mg/g (DW). It was also seen that the withanolide A contents was highest in Poshita stem i.e. 0.252 mg/g (DW) followed by Jawahar-20 root i.e. 0.151 mg/g (DW) while least withanolide A content was found in Chetak leaf i.e. 0.015 mg/g (DW). The withanolide contents were also reported in aerial parts of Ashwagandha as specified in the USP Monograph on Ashwagandha. It was also observed from the study that there was no correlation between the withanolide A contents with respect to different explants of different varieties. The ratio between withaferin A and withanolide A contents was also not consistent with respect to explants or varieties.

On the basis of above data it was observed that out of these varieties of *Withania somnifera*, Poshita recorded higher withanolide contents followed by Jawahar-20. Study of Jawahar-20 and Poshita (*in-vitro* and seed propagated) plants and reported that withaferin A content was higher in leaves in comparison to roots. Seed propagated plants reported higher contents of withaferin A than *in-vitro* while there was no significant difference in withanolide A contents and also confirmed withanolide contents were higher in Poshita as compared to Jawahar-20 [18]. Further on comparing the two promising varieties, Poshita and Jawahar-20 (field grown and *in-vitro* grown) it was seen that the withaferin A contents was highest in Poshita leaf (field grown) i.e. 0.875 mg/g (DW) followed by Jawahar-20 root (field grown) i.e. 0.081 mg/g (DW) while least withaferin A content was found in Poshita root (*in-vitro* grown) i.e.

0.093 mg/g (DW) (Table 1). On the other hand withanolide A contents was found to be highest in Jawahar-20 root (field grown) i.e. 0.149 mg/g (DW) followed by Poshita root (field grown) i.e. 0.082 mg/g (DW) while least in Jawahar-20 leaf (field grown) i.e. 0.021 mg/g (DW). It is reported that greenhouse grown plant contained higher amount of withanolide compared to the *in-vitro* grow plant. It was also seen that in comparison to Jawahar-20, Poshita recorded higher withanolide contents [17]. The conventional propagation is difficult therefore *in-vitro* propagation methods have been developed by many researchers [19]. It was reported that six month old and one year old tissue culture derived plants yielded 0.066 and 1.6% withaferin A respectively, and six month old seed derived plants showed a higher accumulation of withaferin A than tissue culture plants of the same age [20].

A further comparative study of the callus (6 months old) derived from the *in-vitro* grown seedlings (6 months old) the leaf and root explants of the two genotypes *viz.* Jawahar-20 and Poshita were also done (Table 2). It was found that callus derived from Poshita root showed the

highest withaferin A content i.e. 0.256 mg/g (DW) followed by callus derived from Jawahar-20 leaf i.e. 0.198 mg/g (DW), while least withaferin A content was found in callus derived from Poshita leaf i.e. 0.075 mg/g (DW). On the other hand the withanolide A content was found to be highest in callus derived from Jawahar-20 leaf i.e. 0.084 mg/g (DW) followed by callus derived from Poshita root i.e. 0.047 mg/g (DW) while least was found in callus derived from Poshita leaf i.e. 0.036 mg/g (DW). The present study reveals that the production of withanolide/withaferin contents varies from explants to explants and variety to variety. Wiermann [22] suggested the importance of explants selection on the production of withanolides. Culture lines established from leaf explants accumulated the highest level of withanolides and those from shoot tips of well-grown plants produced the lowest level. The varied capacity to synthesize secondary metabolites by *in-vitro* cultures has been attributed to differences in the morphological nature of explants utilized to initiate tissue culture lines. Donor tissues have been an important role to play in the biochemistry of subsequent cultures lines [23-25].



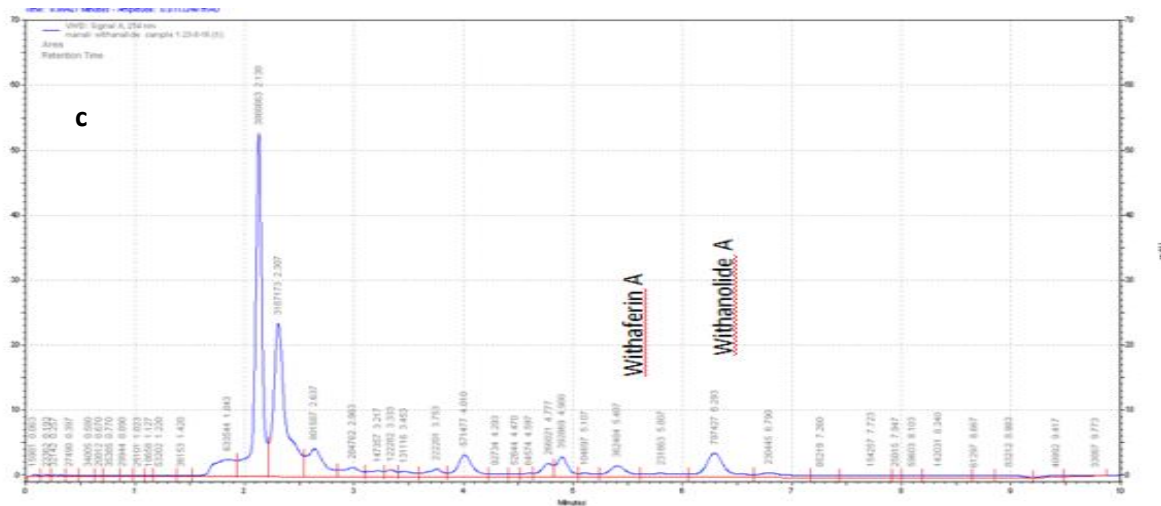


Fig. 1: HPLC Peak profile of (a) Standard of Withaferin A and (b) Standard of Withanolide A and (c) Sample

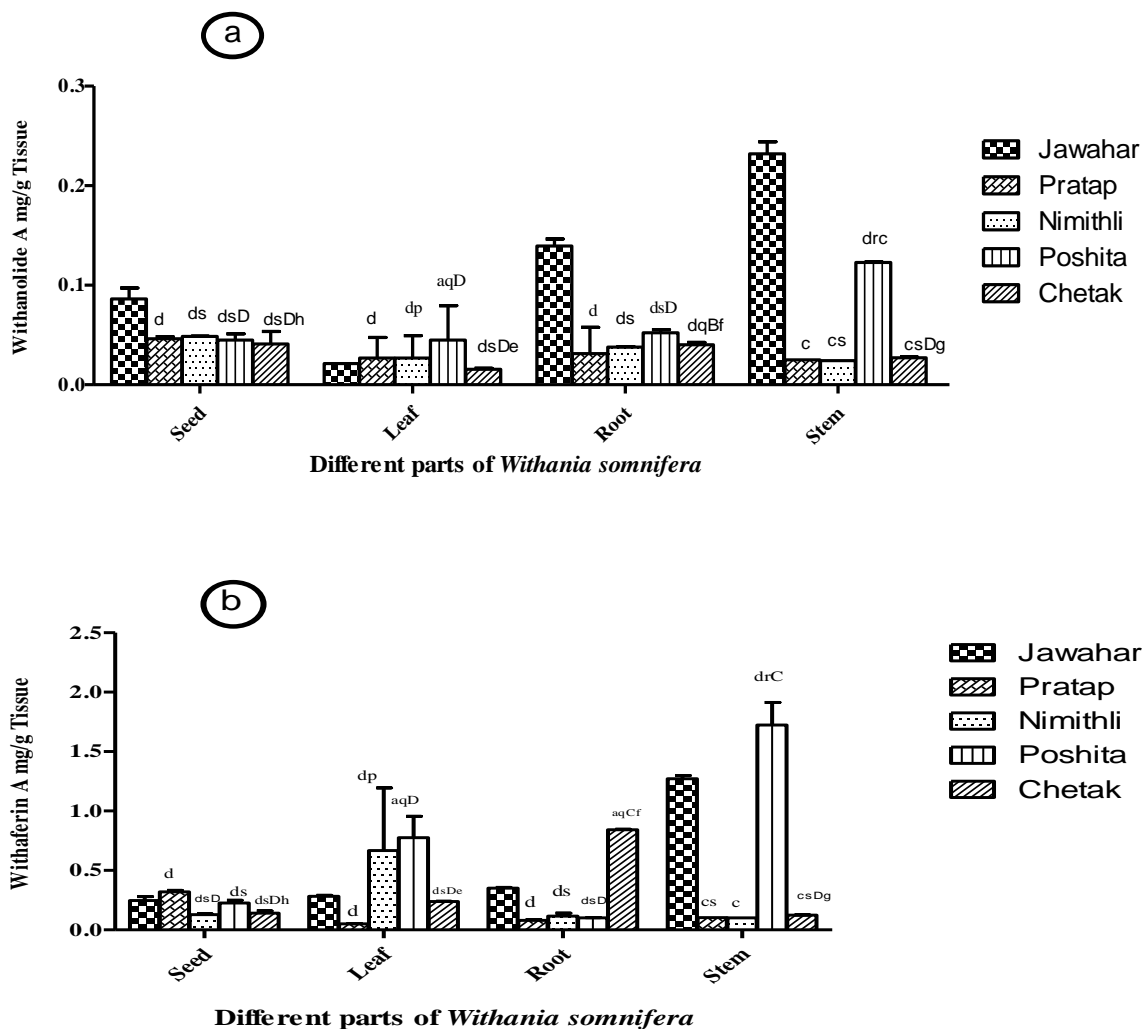


Fig. 2: (a) Withanolide A (b)Withaferin A, in different tissues of *Withania somnifera* varieties(field grown).

All values represent mean±SEM (n = 3). P value versus Jawahar-20: a<0.05; b <0.01; c <0.001; d, Not significant.

P value versus Pratap: p<0.05; q <0.01; r <0.001; s, Not significant. P value versus Nimithli: A<0.05; B<0.01; C<0.001;

D, Not significant. P value versus Poshita: e<0.05; f<0.01; g<0.001; h, Not significant

Table 1: Comparison of withanolide A and withaferin A contents in field grown and *in-vitro* grown tissue

Name of samples	Tissue's Withaferin A (mg/g)		Tissue's Withanolide A (mg/g)	
	Field grown	<i>In-vitro</i> grown	Field grown	<i>In-vitro</i> grown
Leaf				
Jawahar-20	0.282±0.004	0.131±0.007	0.021±0.012	0.085±0.004
Poshita	0.875±0.102	0.097±0.004	0.044±0.019	0.038±0.018
Root				
Jawahar-20	0.081±0.003	0.147±0.002	0.149±0.001	0.030±0.001
Poshita	0.101±0.0004	0.093±0.0009	0.082±0.001	0.040±0.002

CONCLUSIONS

The present investigation was made in the assessment and comparison of withanolide contents in different parts of *Withania somnifera* (L.) Dunal (Ashwagandha) to explore the elite genotype and the possibilities for developing a reproducible protocol for induction establishment and optimization of cultures of *Withania somniferous* for large scale production and to increase the withanolide contents. Thereby promoting the cultivation of elite germplasm in perspective of hill agriculture.

Moreover looking into the fact of extensive utilization for commercial needs of withanolides A/ withaferin A *in-vitro* cultivation of Poshita genotype can offer a more promising approach for the enhanced biosynthesis of the bioactive ingredient and later on their extraction for drug development.

CONTRIBUTION OF AUTHORS- There is no conflict of interest of the authorship. The main work was executed by Manali Singh and Pallavi Shah helped in HPLC analysis. The experiment was planned by Dr. Sanjeev Agrawal.

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