COMPARISON AND DETERMINATION OF WITHAFERIN-A AND WITHANOLIDE A IN DIFFERENT CULTIVATORS OF WITHANIA SOMNIFERA BY RP-HPLC METHOD

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| ARTICLE INFO	ABSTRACT
Key Words *Withania somnifera*, Withaferin A, Withanolide-A	Ashwagandha (*Withania somnifera*) is a significant and highly valued medicinal plant in various indigenous system of medicine (Ayurveda, siddha and unani).In the present study an attempt is made to develop RP-HPLC method for the estimation of important chemical constituents like Withaferin-A and Withanolide-A and their qualitative and quantitative evaluation of Withaferin A and Withanolide collected from different cultivators. This method was reproducible, accurate and buffer free. Several samples collected from different areas and analyzed by HPLC using PDA detector. The analysis was performed on Phenomenex Sphere clone ODS (2) column (4.6 X 250 mm, 5µm particle size) through gradient mode using Acetonitrile and Water as mobile phase for 50 min and spectral acquisition was performed at λ_{max} 227 nm.

INTRODUCTION

Withania somnifera (Linn.) (Family Solanaceae) Dun is commonly known as ''Ashwagandha'', Winter Cherry, Indian ginseng. Ashwagandha is well known for its medicinal properties since the time of punarvasu Atreya, an ancient scholar who taught medicine at Taxila University about 1000 B.C. This plant is used for curing variety of diseases like inflammation [1], anxiety [2], neurological disorders [3], Parkinson's disease[4],hyperlipidemia [5]. Withaferin A and Withanolide A show antitumor and cytotoxic activities [6]. The leaves of this plant were known to act as an insect repellent [7].Ayurvedic texts including Charaka samhita, Susrutha samhitha and Astanghrihadaya (collectively known as Brihatriyi) and Bhava prakash mention ashwagandha to be a general tonic as

Well as cure for morbidity arising from diseases such as pain, arthritis, inflammation. Ashwagandha is mentioned in various Ayurvedic nigantusas tonic, alternative, pungent, astringent, and aphrodisiac and is recommended in rheumatism, cough, dropsy, consumption, and senile debility.Leaves are used for curing fever, lesions, swelling, sore eyes and syphilitic sores. Green berries are used for treating ringworm infection, animal sores and horse's girth galls[8]. The presence of a number of compounds from the roots and leaves of the plant were reported with two new monohydric alcohols, Withaniol, $C_{25}H_{33}O_4OH$ and somnirol, $C_{32}H_{43}O_6OH$, a new dihydric alcohol, somnitol, $C_{33}H_{44}O_5(OH)$, an acidic hydrolytic product, Withanic acid, $C_{29}H_{45}O_6$ COOH, a nitrogen containing component, $C_{12}H_{16}N_2$, Phytosterol, $C_{27}H_{46}O$ and ipuranol,

$C_{25}H_{38}O_2$ $(OH)_2$[9]. Withanolide A which was previously isolated from Withania coagulans has been isolated from roots of *W.somnifera* in 1971 [10].The effects of *W.somnifera* extract (100 or 200 mg/kg, po) against pentylenetetrazol (PTZ) seizure threshold in mice. The drug was tested alone and in combination with exogenous gamma-amino butyric acid (GABA), a GABA receptor agonist or with diazepam. *W. somnifera* increased the PTZ seizure threshold for the onset of tonic extension phase.[11]. Five new Withanolides from the stem bark of *W. somnifera*, collected from the southern region of Delhi, namely Withasomnilide, Withasomniferanolide, Somniferanolide, Somnifera Withanolide and somniwithanolide[12].Three new Withanolides, Withasomniferol A, Withasomniferol B and Withasomniferol C from the non-basic fraction of the benzene and ethyl acetate extracts of the roots of *W. Somnifera* were isolated[13]. Till now very few analytical methods were available for the estimation of chemical constituents of *W. somnifera*. The RP-HPLC method was reported using buffer as mobile phase to separate the marker compounds and covering a broad range of differentially functionalised phytomolecules[16,17,18]. Also, reported methods have more baseline resolution. To overcome above mentioned difficulties we have developed a reliable, reproducible, buffer free and efficient HPLC method involving Photo Diode Array detection (PDA) for the analysis of the two major chemical constituents Withaferin A and Withanolide A from *W. Somnifera*. (Fig.1).[14]
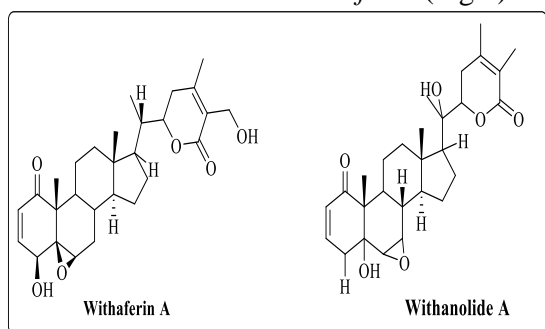
Figure 1: Structures of two standards Withaferin A and Withanolide A from *Withania somnifera.*

Only limited reports are available for simultaneous determination of Withaferin A and Withanolide A from root parts of the plant and there are no comparative studies from different geographical areas.

2. MATERIALS AND METHODS:
2.1: Chemicals and reagents.
The standards Withaferin A and Withanolide A (Fig. 1) were procured from M/S Natural Remedies Pvt. Ltd., Bengaluru, India used as a reference standards for method development and analysis for *W. Somnifera* samples. Methanol and Acetonitrile used were HPLC- grade (Merck, Bombay, India) and were filtered through a Millipore filter (0.5μm) as required. Water used was HPLC grade (Rankem, New Delhi, India).

2.2: Plant Collection
W. Somnifera root samples were collected at different places of India i.e., Ananthapur District of Andhra Pradesh, Gadwal & Kamareddy Districts of Telangana, Utter Pradesh and CSIR-CIMAP, Research Centre, Hyderabad.

2.3: HPLC Sample preparation
One gram of extracted Withaferin-A and Withanolide A from *W. Somnifera* was weighed and transferred into a conical flask. 30 ml of 30% aq. methanol was added and this was extracted by sonication at 45^{0}C for 30 min. Extraction was filtered through filter paper into a 100 ml volumetric flask and the residue was returned to the conical flask. The above extraction procedure was repeated for two more times and made up to the volume with 30% aq. methanol. These samples were analyzed by RP-HPLC method. Each sample solution was filtered through a 0.45μm membrane filter into HPLC sample vial before HPLC injection

2.4: HPLC Analysis
Instruments:
HPLC: water modular system,1524 pumps, model 717 plus injector, model 2996 photodiode array detector, software: empower, column: Phenomenex Sphereclone ODS (2) column (4.6 X 250 mm, 5μm particle size, Made in USA) , Mobile phase: acetonitrile + (solvent A) and water (solvent B) were used for the analysis. All the gradient segments were linear (curve type 6, Waters Empower software). The wavelength range of the PDA detector was set to 200–400 nm and the chromatograms were recorded at 227 nm.

3. Method development

Various mobile phases have been trailed to get the best chromatographic separation among them are isocratic elution of methanol and water, Acetonitrile and water, gradient elution of methanol and water, gradient elution of Acetonitrile and water. Good separations and suitable retention time of two analytes (Withaferin A and Withanolide A) were obtained in gradient elution using Phenomenex Sphereclone ODS (2) column (4.6 X 250 mm, 5μm particle size) with mobile phase consisting of acetonitrile and water. This mobile phase gave the best chromatographic resolution with sharp symmetric peaks. Also for the simultaneous determination of two analytes wavelength range between 210-300nm was used. Good signals were observed only when the wavelength was set at 227 nm for Withaferin A and Withanolide A.

3.1. Method validation: The proposed chromatographic method was validated according to ICH Q2(R1) guidelines.

Accuracy: Accuracy was assessed by standard addition method, standard was added at three different levels (50%,100%.150%)to the sample solution.

Linearity: Three replicates of 2-20 **μg**/mL concentration range of Withaferin A and Withanolide A. Injected for linearity study.

Specificity: Specificity method was ascertained by separation of analyte from other potential components such as impurities, degradants or excepients. A volume of 20 **μ**L of placebo solution, Withaferin A and Withanolide A standard solution, sample solutions was injected and chromatogram was recorded.

System suitability: System suitability test was performed by injecting six replicates of Withaferin A and Withanolide A working standard solution 100 μg/ml of concentrations and observe he parameters are tailing factor, theoretical plates and percentage relative standard deviation (RSD %) of peak area

Robustness: The robustness was assessed by altering the optimized chromatographic conditions such as by changing the flow rate, the mobile phase composition and wavelength.

Precision: The method of precision was determined by repeatability and reproducibility. The repeatability of instrument was checked repeatedly injecting and analyzing (n=5) standard solutions of Withaferin A and Withanolide A(10 μg/ml). Intermediate precisions include intraday and interday precision. The intraday and interday precisions method was analyzed by three sets of different concentrations (2,10,20 μg/ml) on the same day and on three different days.

Limit of Detection and Limit of Quantification:

Limit of detection (LOD) and limit of quantification (LOQ) were determined by using the formula based on the standard deviation. LOD and LOQ were calculated by using the formula LOD=3.3x σ/S and LOQ= 10xσ/S where σ is standard deviation and S is he slope of corresponding calibration curve.

4. RESULTS AND DISCUSSION:

Method optimization was achieved on Phenomenex Sphereclone ODS (2) column (4.6 X 250 mm, 5μm particle size) column using Acetonitrile and water in gradient mode. Two peaks (Withaferin-A& Withanolide-A) were obtained at 26.61 and 29.67minusing flow rate 1.0 ml/min respectively, as shown in Fig. 2. The optimized method was validated as per ICH guidelines. % RSD of peak area (Withaferin-A& Withanolide-A) was found to be 0.89&0.86 theoretical plate was more than 2000 which showed that the instrument is suitable for further validation of parameters. The calibration curve (Withaferin-A& Withanolide-A) was achieved to be linear over a range of 2-20 μg/ml with regression coefficient of 0.9993 & 0.9882. The proposed method was found accurate with 99.83 % , 100.64 % recovery(Withaferin-A& Withanolide-A).The % RSD values of (Withaferin-A& Withanolide-A) repeatability (0.28 %,0.26%) inter-day (0.05-1.63 %,0.08-1.82) and intra-day (0.16-1.65 %,0.18-1.98) variations revealed that the proposed method has good precision. The LOD and LOQ values (Withaferin-A& Withanolide-A) were found to be 0.05 μg/ml,0.07 μg/ml and 0.16 μg/ml,0.18 μg/ml respectively. The method was found to be robust with change of ±2 % in wavelength, flow rate and mobile phase ratio. In specificity study, there was an absence of interference from the other compounds, showed that method is specific. The proposed method can be used for the routine analysis for the estimation of Withaferin A and Withanolide A.
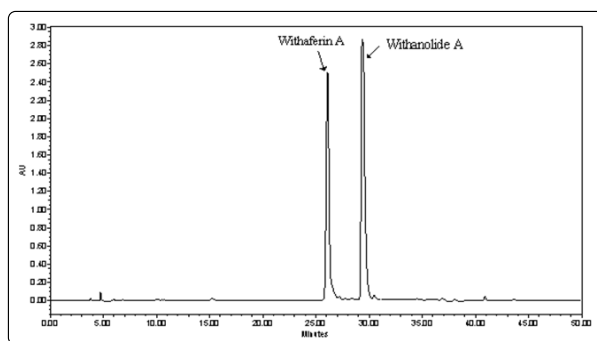
Figure 2: HPLC chromatogram of standards.

Table 1: Variation of Withaferin A and Withanolide A from different cultivars

S.No.	Collection Area	Percentage (%)	
		Withaferin-A	Withanolide-A
1	Maldakal (M) Gadwal (D)-I	0.046	0.075
2	Undavally (M) Gadwal (D)-I	0.049	0.077
3	Ayija (M) Gadwall (D)-II	0.119	0.187
4	Maldakal (M) Gadwal (D)-III	0.023	0.033
5	Undavally (M) Gadwal (D)-IV	0.053	0.082
6	Raja pet (Vill & M) Kamareddy (D)	0.319	0.359
7	Undavally (M) Gadwal (D)-V	0.082	0.099
8	Undavally (M) Gadwal (D)-VI	0.033	0.063
9	Undavally (M) Gadwal (D)-VII	0.024	0.020
10	Undavally (M) Gadwal (D)-VIII	0.053	0.050
11	Undavally (M) Gadwal (D)-IX	0.036	0.045
12	Guntakal, Ananthapur	0.119	0.138
13	NIMETLI-101	0.050	0.380
14	Uttar Pradesh –I	0.047	0.067
15	Uttar Pradesh –II	0.020	0.098
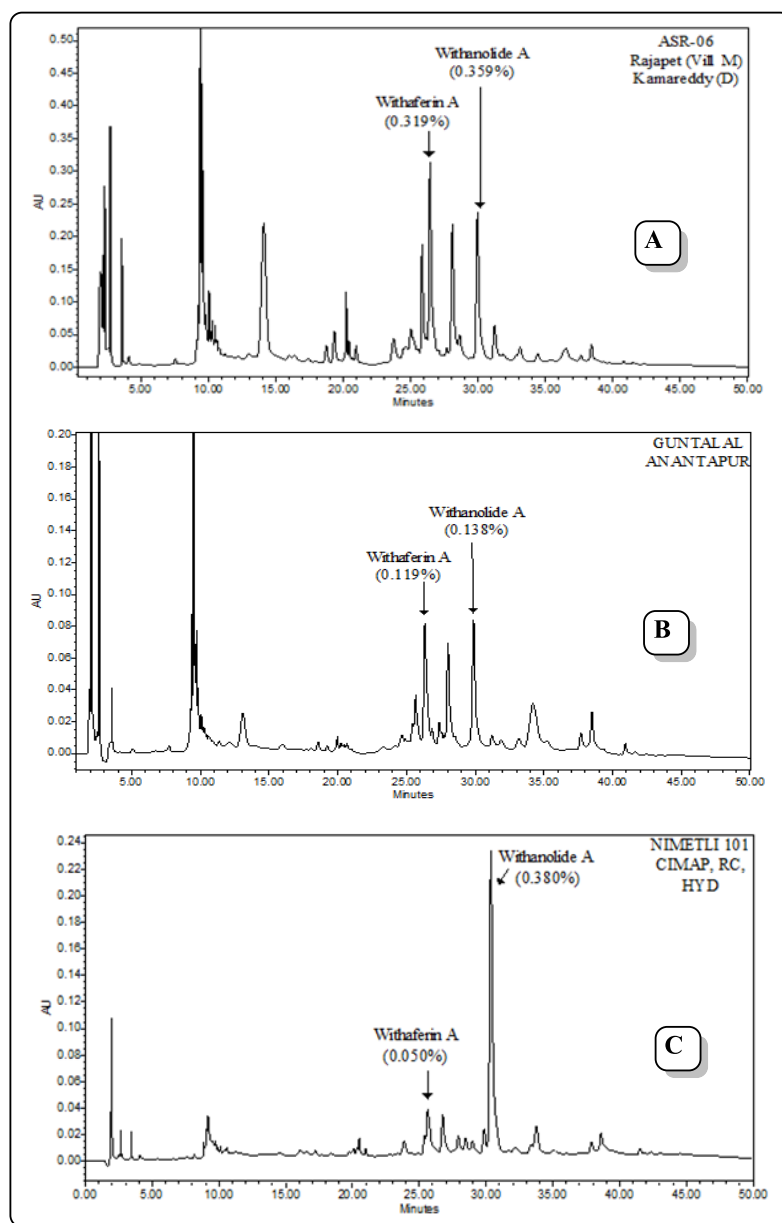
Figure 3. HPLC chromatograms of (A) Rajapet (Vill & M), Kamareddy (Dist), Telangana state (B) Guntakal area of Ananthapur, Andhara Pradeshand (C) NIMETLI 101 roots respectively.

5. *Comparative studies*

Chemical analysis based on quantification of two major bioactive molecules from roots of *W. Somnifera* revealed a significant chemical variability among the collected populations as depicted in Table 1. CIMAP developed *W. Somnifera* variety Poshita cultivating different areas of India and the differences in qualitative and quantitative analysis were observed between various cultivators. The marker compounds Withaferin A ranging from 0.02 to 0.319% and Withanolide A 0.02-0.359% were obtained. Whereas another CIMAP variety NIMETLI-101 contains high percentage of Withanolide A

(0.380%). Fig.3A shows the representative HPLC chromatograms of Rajapet (Vill & M), Kamareddy (Dist.), Telangana state of India of *W. Somnifera* root. The marker compounds both yielded similar amounts (0.319 and 0.359% respectively). That geographcal conditions are highly suitable for cultivation of withaferan A and withanolide A . Whereas high rain defficiency area of India i.e Guntakal area of Ananthapur, Andhara Pradesh has also contains considarable percentage of withaferin A and Withanolide A (fig: 3B: 0.119 and 0.138% respectively). Fig 3C shows the respective HPLC chromatogram of CIMAP high yiled variety NIMETLI-101 root and

observed high percentage of Withanolide A content (0.380%). The least percentage were observed in Uttar Pradesh samples (withaferan A ranging from 0.020 to 0.047% and withanolide A ranging from 0.067 to 0.098%).

6. CONCLUSION:

The developed method was significant for the analysis of Withaferin A and Withanolide A in *W. somnifera*. It is not only allows determining in the root material also it is suitable for the quantification of standard in leaf and stem parts of the plant. Thus, it is helpful for scientific as well as commercial applications. The comparative studies done for variations of Withaferin A and Withanolide A from *W. Somnifera* collected from different places of India i.e., Gadwal ,Ananthapur District of Andhra Pradesh, Kamareddy Districts of Telangana, Uttar Pradesh and CSIR-CIMAP, Research Centre, Hyderabad . Kamareddy district area sample contains more percentage of Withaferin A (0.319%) and Withanolide A (0.359%) compare to other regions. Similarly CIMAP developed variety NIMETLI-101 collected at CIMAP, Research Centre; Hyderabad root sample contains more percentage of Withanolide A (0.380%).Thus, Kamareddy area was suitable for cultivation of Withanolides rich *W. Somnifera.*

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