Analysis of Ashwagandha (*Withania somnifera*) alkaloid by HPLC methods

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Abstract

Withania somnifera (Family; Solanaceae) is commonly known as Ashwagandha and is used in traditionally system of medicine since long time. Many compounds have been reported from the root part of the plant. In present study, comparative evaluation of microbial consortium and uninoculated control plant root collected from different geographically sources was done by HPLC. Methanol soluble root extract of the plant was subjected to Column chromatography to isolate and purify the phyto-constituents which can be termed as markers. Three compounds were isolated and purified. Compounds were characterized as withanone and withaferin-A. The third compound was found to be impure. Novel HPLC methods were developed for assessment of purity, for standardization and for estimation of the compounds. Simplicity of isolation and HPLC analysis for the compounds suggests that the compounds may be termed as markers for the standardization of the methanol extracts and preparations containing *Withania somnifera*.

Keywords: *Withania somnifera*, phyto-constituents, HPLC and Withaferin - A

1.INTRODUCTION

Withania somnifera, also known as Ashwagandha, Indian ginseng and winder cherry, has been an important herb in the Ayurvedic and indigenous medical systems for 3000 years. Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory bronchitis, asthma, ulcers, emaciation, insomnia and senile dementia. Clinical trials and animal research support the use of Ashwagandha for anxiety, cognitive and neurological disorders, and inflammation and parakinsons disease. Ashwagandha chemopreventive properties make a potentially useful adjunct for patients undergoing radiation and chemotherapy. Ashwagandha is also used therapeutically as an adaptogen for patients with nervous exhaustion, insomnia and debility due to stress and as an immune stimulant in patients with low white blood cell counts. The major biochemical constituents of Ashwagandha root are steroidal alkaloids and steroidal lactones in a class of constituents called withaferin-A. For the preparation of methanol and aqueous extracts from root, stem and leaves conventional methods like sonication, refluxation, percolation and counter current extractions are used. Various Withaferin - A isolated from root parts of *Withania somnifera* have been analysed (Ganzora *et al*., 2003). Withaterin-A are present in roots, it is medicinally used. In the present investigation an attempt was made to isolate chemical constituents from the root of *Withania somnifera* and characterized them as markers and chemical comparison of them with the roots by HPLC (Dalavayis *et al.,* 2006) fingerprinting. To improve the commercial outlook for the production of Withanterin-A, advanced chromatographic techniques were used. The present study helped in the

isolation of Withanterin-A, from roots leaves and the yield chemical entities with that present in the root of *Withania somnifera.*

2.MATERIALS AND METHODS

2.1 Ashwangandha root sample

Fresh root of *Withernia somnifera* (Ashwangandha) were collected in Department of Microbiology Garden Faculty of Agriculture, Annamalai University, Annamalainagar the collected root were dried at room temperature and then grounded into uniform powder. Solvent Methanol of A.R and HPLC grade purchased from E-merck for extraction and analysis.

2.2. Preparation of standard and samples for HPLC analysis Standard

10 mg of Withaterin-A working standard was dissolved in 50 ml of methanol (HPLC grade) which was further diluted by dissolving 1 ml of this solution to 50 ml methanol.

2.2.1. Sample preparation

1 mg of the root samples were accurately weighed and dissolved in 50 ml of methanol HPLC grade. Further diluted 1 ml of this solution to 50 ml using methanol HPLC grade. 20ml of standard and sample were injected to HPLC and record the chromatogram, calculated the content of Withaterin-A of the samples in comparison with standard.

2.2.2 Analytical method

HPLC estimation of Withaterin-A performed on Shimdzu 10 AS HPLC system, equipped with UV detector. For estimation of Withatexin-A, Lichosorb C18 RP column (250×4.6 mm, µm particle size) and the mobile phase with mixture of acetonitrile: Methanol: ortho phosphoric acid (55:45:1) For peak confirmation as GC-MS experiment using an AQA mass spectrometer from finnigan together with a Finnigan HPLC (As 3000 auto sampler, P4000 pump and UV5000 Lp detector) was performed. Best results were obtained in positive ES1 mode, with ionization voltage set to 50 V source voltage to 3.0 Kv and probe temperature to 350ºC with this instrumentation the separations had to be performed at ambient temperature. Thus in order to obtain similar retention times and good detection sensitivity the flow rate was reduced to 0.5 ml/min and the solvent gradient modified (55 A/ 45 B to 45 A/ 55 B in 25 min).

Accuracy

A recovery experiment was performed to confirm the accuracy of the method. Sample withaferin-A (1.0g) was spiked with 1.00 ml of the standard stock solution, and the extracted and analysed under optimized conditions. The recovery rates obtained were 97.59 % for 1 and 100.0 % for 2.

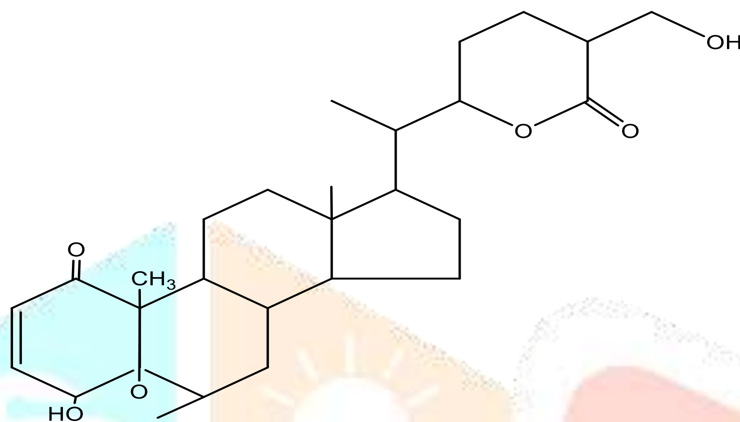
Ruggedness

Intra and inter-day assay precision of the method was determined by analyzing five individual sample of one specimen (sample withaferin-A) on three consecutive days. The sample were extracted and assayed under optimized conditions; for detailed results see Table 2.

3. RESULTS AND DISCUSSION

The separation of three methanolic *Withania somnifera* extracts (root) under optimized conditions in shown in Fig 2. A biologically active Withaferin-A which are also commercially available, where chosen as marker compounds of similar polarity in less than 25 min after all separation parameters were carefully.

Fig. 1 structures see Withaferin-A

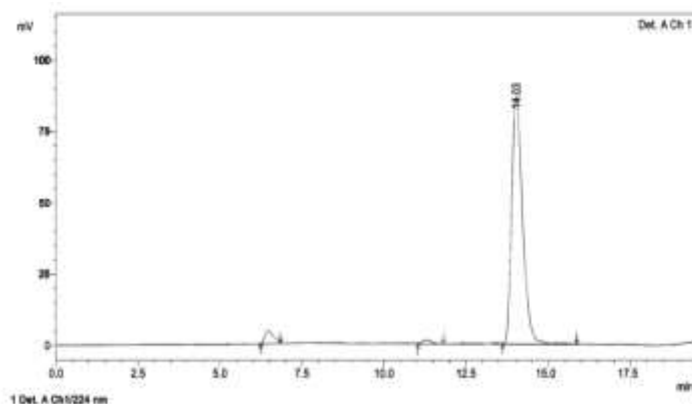


Molecular formula = $C_{28} H_{38} O_6$
Molecular weight = 470.598g/mol
Melting point = 241 to 245°C

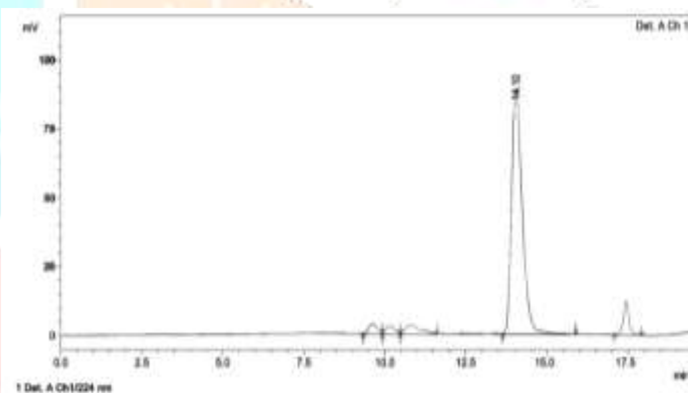
3.1.Chemical structure of Withaferin –A

Stationary phase of several manufacturers with different polarity (C-8, C-12, phenyl hexyl, particle size 3-5 µm) and column length (10-25cm) were initially screened for their applicability. A synergi C18 column (C-12 phase, 4µm particle size, 250×4.6 mm) from phenomenex showed the best results regarding peak symmetry and separation selectivity. The right mobile phase was crucial for a satisfactory result as well only by using methanol compound Withaferin-A could be well resolved from signal of the unidentified substance (a) (Fig. 1). In combination with reagent alcohol the separation was further improved, whereas the addition of acid, abutter or a modified (MBE, THF) to the mobile phase was not advantageous. Performing the separation time and the column backpressure, without any decrease in peak resolution. Additionally, an GC-MS experiment was performed to confirm the 3WQpeaks of interest (Fig. 3). The HPLC conditions were slightly changed as the separation had to be performed at room temperature and flow rate of 1.0 ml compounds with modifications in the solvent composition and flow rate, the MS signals were readily assignable. In positive ESI mode, the spectra of 1 showed signals at m/2 of 488.3 (M + N H4)+. The method was validated in accordance to USP determining, several analytical and statistical parameters. Linearity of the detector response for the two standard compounds was confirmed between 400.0 and 1.6 µg/ml, with a detection limit of at least 0.26 µg/ml (see Table 1 for the exact data). Peak purity and identify were verified by studying the PDA and MS data, as well as by spiking samples with reference compounds, no indications of impurities could be found. Accuracy of the method was confirmed by performing a recovery experiment. Sample Withaferin -A was spiked with a known amount of the authentic to the theoretical amount, recoveries off 97.59% for Withaferin-A were obtained. All standards and samples were injected in triplicate. The resulting R. S. D. of less than 2.0 % confirmed the

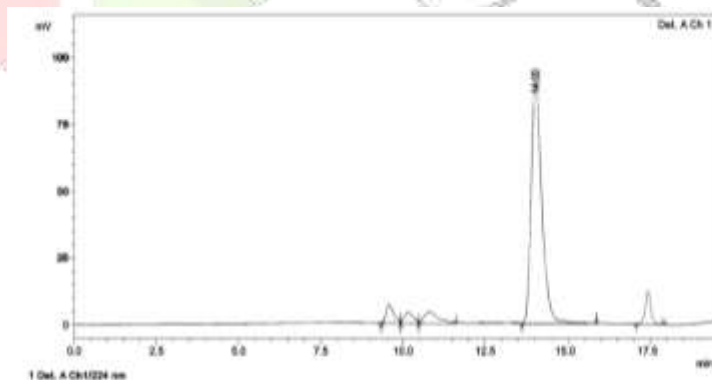
precision of the method. Intra and inter-day variation of the assay was determined and showed to be lower than 5.0 % with a maximum R.S.D of 4.86 % reached at day 3 for compound Withaferin-A. The inter-day precision was better than 2.0 % for all compounds (Table 2).



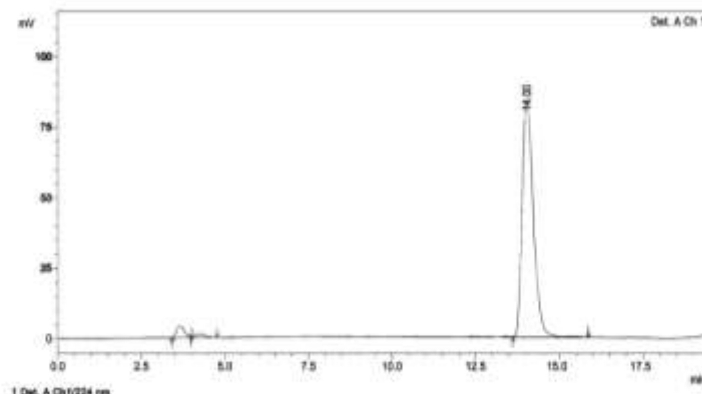
Single Inoculation *Azospirillum.sp*



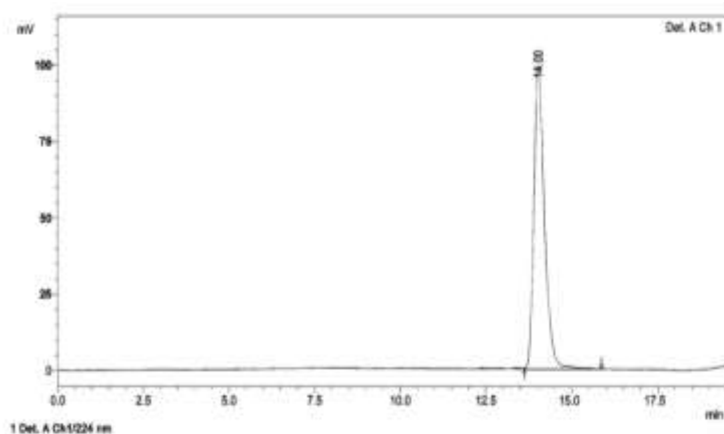
Double Inoculation *Azospirillum* + *Pseudomonas fluorescence*



Consortium = *Azospirillum* + *Azotobacter* + *Bacillus* + *Pseudomonas fluorescence*



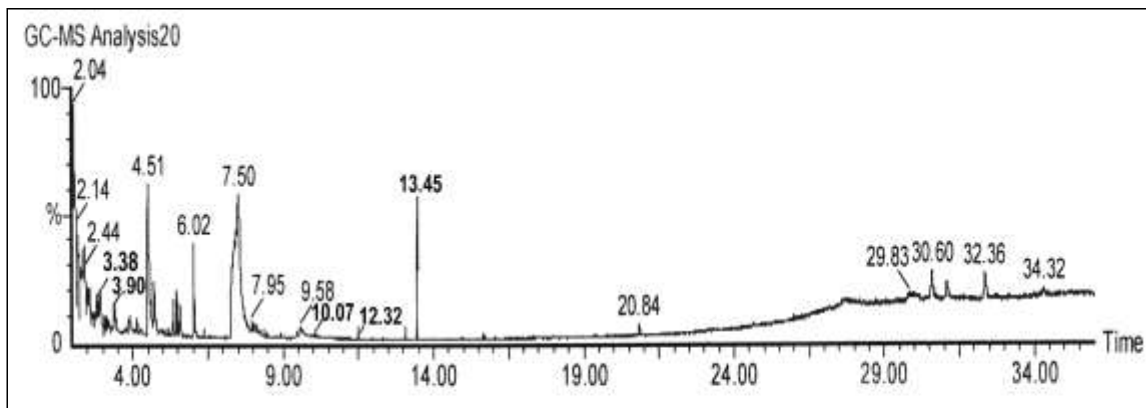
Uninoculated control



Standard

Fig 2. HPLC Estimation of Withaferin - A from *Withania somnifera* plant root sample

Prior the analysis of Ashwagandha sample and products, the efficiency of the extraction procedure was verified. Thus one sample Withaferin-A was repeatedly extracted with 3 ml of the methanol and extract analyzed separately. Compounds Withaferin-A were not detectable after the fifth repetition of the procedure. A minimum of 97.8 % of the compounds toes in solution after a 3 fold extraction, therefore, this was already considered as an exhaustive procedure. The analysis of *Withania somnifera* microbial consortium and uninoculated control plant root confirmed the presence of Withaferin- A in root parts, of the plant, but with significant differences in their ratio (Table 3). This compound was only minor in uninocualted control plant root were a consortium rather high amount of Withaferin-A was found (0.238 %). Single and Double inoculated contained the lowest percentage of total Withaterin-A 1 and 2 (0.055 %). Finally six market products (capsules, tablets and liquid extract containing *Withenia somnifera*) were analyzed. In all of these, even in multi component preparations. The one marker compounds were assignable pot all of the samples followed the previously described pattern with Withaferin-A being in roots. The higher percentages of Withaferin-A samples. A different extract procedure applied by the manufacturer of chemo types of the plant (which have been reported in the literature (11, 14)). The content of Withaferin-A in solid samples varied from 0.009 % to 0.100% comparing the total daily uptake, based on the manufactures suggestions, permits a better estimation at the overall product quality. As it is evident from Table 3, values from 0.06 to 2.57 µg/day were obtained which is nearly a 4 – fold variation.



GC MS chromatogram of *Withania somnifera* root samples

Table 1: Correlation coefficient (R2), regression equation and limit of detection (LoD) for compound Withaferin-A

Compound	**R^2**	**Regression equation**	**LoD (µg/ml)**
Withaferin -A	0.9996	$Y=1.25\times10^4\times$	0.26

Table 2: Intra and inter day precision of sample Withaferin-A assayed under optimized conditions

Compound	**Intra – day***			
	Day 1	**Day 2**	**Day 3**	**Inter – day****
Withaferin – A	353.81 (4.74)	358.96 (2.85)	346.54 (4.86)	353.10 (1.76)

Samples in µg/g; R.S.D's are given in parentheses; * (n=5); ** (n=3)

Table 3: Percentage of withanolides in different *Withania somnifera* samples

Sample	**Description**	**1**	**2**	**1-2 §**
W.S. 1	Single inoculation (*Azospirillum*)	0.035 (1.04)*	0.019 (1.61)*	2.51
W.S. 1	Double inoculation (*Azospirillum,Pseudomonas fluorescence*)	0.027 (1.04)**	0.238 (1.52)**	1.32
W.S. 1	Consortium (*Azospirillum, Azotobacter, Pseudomonas fluorescence* and *Bacillus*)	0.065 (1.02)**	0.364 (1.06)**	2.57
W.S. 1	Control plant	0.066	0.193	-

		(1.91)*	(1.96)*	

*Values in g/100g; ** Values in mg/ml; n=3, R.S.D. s are given in parentheses; §, daily uptake of withanolides in commercial samples (mg/day).

4. CONCLUSION

In conclusion, the method described herein represents a significant improvement in the analysis of alkaloid in *Withania somnifera.* In not only allows the direct, rapid and accurate determination of withaferin-A in plant material but it also fulfills all criteria of a validated method. Thus it should be helpful for scientific as well as commercial applications.

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