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HPTLC Quantification of *Withaniasomnifera* (L.) Dunal

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Abstract
At present study a simple, selective, easy, robust, sensitive and precise method is developed to investigate the outcomes of HPTLC for the simultaneous determination of 3 key withanolides, namely withaferin A, withanolide A and withanolide B in Ashwagandha; Withaniasomnifera plant part samples. The method is analyzed simultaneously on Camag aluminum per-coated silica gel 60-$_{F254}$ HPTLC plates, using toluene: ethylacetate:formic acid:ethanol (6:3:0.1:0.6 v/v/v/v) as mobile phase. The Quantitative evaluation of withanolides was performed by densitometry in the reflection-absorption mode at 530 nm. The method was validated for recovery, precision, accuracy, robustness, limit of detection, limit of quantitation, and specificity conferring to International Conference on Harmonization guidelines. Specificity of quantification was confirmed using retention factor (Rf) values, UV Visual spectral correlation, and electrospray ionization mass spectra of marker compounds in sample tracks. This combination of techniques has been applied, for the first time, for the estimation of withaferin-A, withanolide A and withanolide B in fruit, leaf, stem and root parts samples of Withaniasomnifera. Present method may be beneficial in a quality control setting as it would allow rapid qualitative analysis of herbal material while sustaining accurate quantification of extract composition and can be used for routine analysis as well as for quality control of raw materials and herbal formulations.

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INTRODUCTION

The family Solanaceae has valuable medicinal properties due to the presence of tropane-alkaloids and is the third economically important plant family and ranks the first in terms of vegetable crops, includes many important crop plants like eggplant, tomato, tobacco, potato and pepper. The genus *Withania* Pauquy is distributed throughout tropical and sub-tropical regions of the world with 26 species. The genus is also well distributed in India with 2 species i.e., *W. somnifera* and *W. coagulans*, and is grown well in drier parts of tropical and sub-tropical regions extending to the elevation of 1,500 m [3]. Both the species are well distributed in East of Mediterranean regions extending to South Asia. *Withaniasomnifera* (L.) Dunal., has a reputation in the Indian and African system of traditional medicine and widely used as a home remedy for several diseases in India and other parts of the

world. Both the species are well exploited for their pharmacological. The genus holds an important place in the Indian Ayurvedic system of medicine because of its valuable pharmaceutical and nutritional properties. Indian ginseng or *Ashwagandha* or winter cherry, is one of the most valued medicinal plants of Indian Ayurveda since 3000 years [1] and officially known as Indian Pharmacopoeia [2]. *W. somnifera*, commonly known as '*Ashwagandha*', and in Ayurvedic manuscripts as "Charak Samhita," "Sushrut Samhita," "Astanghirdaya,"and"Bhava-prakash". It is the more exploited species and is very well studied during the past years, commonly used in different forms like ointments, infusions, decoctions, powder or syrup [3]. The composition are mainly alkaloids and sterollactone based phytochemicals, like ashwagandhine, cuscohygrine, isopelletierine, anaferine, anhygrine, tropine, sitoindosides (saponins), the diversely functionalized withanolides, withanamides, and glycowithanolides have been isolated from different parts of this plant [4, 5]. Of all the phytochemicals reported, withanolides of which withaferin-A attributes to show most beneficial pharmacological. These compounds are structurally diverse steroidal compounds with an ergosterol skeleton in which C-22 and C-26 are oxidized to form δ-lactone [6]. The importance of this Indian medicinal plant is highlighted with a growing number of explorations on various aspects of drug discovery, and its demand in the global market as a positive health promoter is growing. The increasing therapeutic benefit of both the species of the genus has attracted the attention of pharmacologists for biomedical investigations on plant extracts and isolated phytochemicals.

EXPERIMENTAL

Plant material

The plant material was collected in the months of September–October 2014 from different localities of Udaipur (Rajasthan). Voucher specimens have been identified by BSI Jodhpur and submitted in Herbarium of Punjabi University, Patiala with accessions number as 60576 (P-I), 60577 (P-II), and 60578 (P-III).

Chemicals and Preparation of plant extract

The plants parts i.e., fruit, leaf and root samples were separated washed and dried at room temperature (25°C-30°C).

All the organic solvents as well as standards of analytical grade, used in the present study were purchased from MERCK (Darmstadt, Germany). Solutions of standards were prepared by using 1:1 methanol. The powdered plant parts of each sample were dissolved in 20 ml of aqueous methanol for overnight. The extract was concentrated and dried using rotary evaporator under reduced pressure. 2 mg of each dried extract was again dissolved in 2ml of methanol to obtain 1mg/1ml concentration and stored at 4°C till further analysis.

Chromatography instrumentation and conditions

For the acquisition, a CAMAG HPTLC system comprising a Linomat-V automatic sample applicator and CAMAG TLC Scanner III with win CATSsoftware was used. For the reflectance/absorbance mode deuterium and halogen tungsten lamp is used with high pressure mercury lamp as reflectance/fluorescence mode. A CAMAG 100µL precision syringe from Hamilton, Bonaduz, Switzerland was used for sample application under gentle stream of nitrogen. CAMAG aluminium per-coated silica gel plates were used. Silica gel 60_{F254} plates with 200µm thickness × 5µm particle size from Merck, Dermostst, Germany were used. For the plate development a CAMAG twin-trough chamber 20cmW×10cmH was used. The plate was left in pre-heated oven at 105°C for 5mins. Violet color spots of the standard and the samples appear. The densitometry chromatographs of the plates were taken at different wavelength. *p*-Anisaldehyde sulfuric acid was prepared by dissolving, 5ml of *p*-anisaldehyde solution in 1ml of 98% sulfuric acid and 50 ml of acetic acid. After development and derivatization of the plate, measurements were made by winCATS software. Concentration of the target analytes in the separated bands were determined from the intensity of the reflected light indicated and the peak areas produced were correlated to the analyte concentrations using six level linear calibration curves. The employed statistical

analysis ensures that the developed method is reproducible and selective. This method can be used as an important tool to ensure the therapeutic dose in herbal formulations, standardization and quality control of bulk drugs.

Chromatgraphic separation

Each extract of 5 µL *Withaniasomnifera* extracts olution was spotted on the HPTLC silca gel plate, 4mm band length, using a Camag ATS4 automatic TLC sampler spotting device. The TLC plate was developed in ascending mode in a twin trough chamber pre-saturated for 30 mins with particular mobile phase. Linear ascending plate development was performed until a migration of distance 8cm from the origin was reached. The plate was removed from the chamber, air dried, derivation with *p*-ansaldehydesulphuric acid, heated, and scanned in absorbance/reflectance mode of a Camag TLC scanner 3 (Figures 1 & 2). Peak area data were recorded using Camag Win CATS software.

METHOD VALIDATION

Method validation involves the performance valuation of various parameters by following the guidelines of International Conference on Harmonization (ICH) [7], and International Union of Pure and Applied Chemistry (IUPAC) [8].

(a) Calibration Curve and linearity

Calibration curves were constructed by plotting the peak areas versus concentration of withanolides (bioactive standards). The regression equations were calculated using the least squares method in Graph PAD Prism 3.0. (Figure 3). Aliquots (Stock solutions of withaferin A, withanolide-A and withanolide B were prepared in methanol and different amounts) of the bioactive standard working solution (2, 4, 6, 8, 10 & 12 µL) were applied in were loaded onto a TLC plate, using ATS4for preparing six points calibration curves (triplicates). Concentration of the target analysts in the separated bands were determined from the intensity of the reflected light indicated and the produced peak areas were correlated to the analysts concentrations using six level linear calibration curves. The regression equation and correlation coefficient were from calibration curves, for withaferin-A, Y = 555.8*X + 307.8, withanolide A, Y = 470*X + 359.4and withanolide B, Y = 470*X + 319.4, Table 1.

Table 1: Data showing different parameters analysised for the reference compounds

Reference compound	**Withaferin A**	**Withanolide A**	**Withanolide B**
Working concentration	2-10µg/Band	2-10µg/Band	2-10µg/Band
R*f* value	0.81	0.45	0.64
Regression equation	Y = 555.8*X + 307.8	Y = 470*X + 359.4	Y = 470*X + 319.4
Correlation coefficient (r^2)	0.998	0.996	0.995
LOD (ng)	260	405	488
LOQ (ng)	788	1228	1479

(b) Accuracy: The accuracy of the method was analyzed by calculating recoveries of withaloides by the standard addition method. Prequantified extracts of plant samples were added to known amounts of standards solutions of the withanolides in triple concentration rages (66, 132 & 198 µg/mL). These values of regression equation of the calibration graphs helped in estimation of withanolides.

(c) Method precision (repeatability) - The instrumental precision of the HPTLC method was checked by repeated spotting on the silica gel 60F254 plate and analyzed with the proposed method and densitometric scanning. It was performed by spotting six samples of same concentrations each from the stock solutions of withaferin-A, withanolide-A and withanolide B

(1,600 ng). Six different samples of same concentrations were spotted on a plate and analyzed by the proposed method to conclude variation arising from method itself.

(d) Intermediate precision (reproducibility): The inter and intraday variations of the proposed method were reassessed by estimating the corresponding responses in triplicate on the same day and on three different days over a period one week.

Sensitivity of the method

Limit of Detection and Limit of Quantitation
Sensitivity of the HPTLC method i.e., the limit of detection (LOD) and limit of quantitation (LOQ) were determined for withanolides using linear regression equation (Table 1) applying equation:

LOD=3Sy,x/b and LOQ = 10Sy,x/b

Where, Sy, x is the standard deviation of the Y-value distribution around the regression line and b is the slope of the calibration graph.

Specificity
The specificity of the method was ascertained by analyzing standard compounds and samples. The spots for standards in samples were confirmed by comparing the R*f* and spectra of the spots with that of the standards. The peak purity of all standards were assessed by comparing the spectra at three different levels, i.e, peak start, peak apex, and peak end positions of the spot.

Recovery
The recovery of the method was determined at two levels i.e., 50% and 100%, by adding a known amount of particular standard to the extracts of plant part, and the mixtures were analyzed by the proposed method.

Ruggedness
The ruggedness of the proposed method was studied using reagents from different lots and different manufacturers.

RESULTS AND DISCUSSIONS

In the present study, withaferin A, withanolide A and withanolide B are simultaneously separated from the mixture at similar experimental conditions (Table 2). The selection of mobile phase plays a vital part in development of any HPTL method, in the present study the mobile phase was selected by performing various systems wherein the mobile phase of different combination of ratios and polarity of solvents with the stationary phase silica gel 60F254 has been experimented. Until a mixture of solvent containing, toluene: ethylacetate: formic acid: ethanol (6:3:0.1:0.6 v/v/v/v) was found suitable for the successful separation of Withaferin A, Withanolide A and Withanolide B. A decent sensitivity, precision, and accuracy were achieved for densitometric quantification of the target withanolides at 530 nm. Satisfactory resolutions of the components (Fig. 2) with pure scan peaks were attained. After successfully running the plate the *p*-anisaldehydesulphuric acid is used as derivatizing reagent for enhancing the compounds and lastly the scanning of the plate is accomplished at 530nm. Peaks corresponding to withaferin-A, withanolide-A and withanolide-B were recorded at R*f* 0.81, 0.45 and 0.64, respectively. The methanolic extracts of fruit, leaf, stem and root of *W. somnifera* was subjected to HPTLC. After running the plate with the mobile phase it showed the presence of withaferin-A, withanolide-A and withanolide-B peaks in all the samples (Fig. 1& 2). The ultraviolent spectral showed the characteristic of the peaks for standards and discovered the identity of withaferin-A, withanolide-A and withanolide-B present in all the plant parts (samples). The calibration curves were linear in the range of 2000, 4000, 8000, 10,000, 12,000, 24,000 ng for withaferin-A, withanolide-A and withanolide-B. Peak purity test was conducted by comparing UV visible spectra of standards and samples tracks. The results showed the highest content of all the three marker compounds in leaf samples. Withaferin-A (94.36±0.12 mg/g of DW) displayed in track 8, withanolide-A (63.89±0.18 mg/g of DW) and withanolide-B (14.27±0.89 mg/g of DW) (Fig. 1 & 2).

Table 2: Data showing bioactive markers, composition of solvent system, derivatizing reagent used and wavelenght of all the marker compounds used in the present study for HPTLC analysis.

Sr. no	Bioactive compound	Solvent system	Composition (v/v/v/v)	Derivatizing reagent	Wavelength (nm)
1.	Withaferin A	Toluene: Ethylacetate: Formic: Ethanol	6:3:0.1:0.6	*p*-anisaldehydesulphuric acid	530
2.	Wthanolide A	,,	,,	,,	530
3.	Withanolide B	,,	,,	,,	530

Largely, the roots of *Withaniasomnifera* are considered to be rich in bioactive withanolides, used in polyherbal preparations [9, 10, 11]. However, in the present study it was observed that leaf samples contained the highest amount of all the three standard compounds (Fig. 1), which are renewable source which is inlegalization with an earlier report [12].

Previously a few reported are recorded in the literature regarding the successful separation of compounds from *Withaniasomnifera* like, a Novel HPTLC method developed for the estimation of various chemotypes [13].As per previous literature a very few thin layer chromatographic (TLC) methods are available for the quantification of withanolides [14, 15, 16], especially for withaferin A [17, 18, 19], however a larger number of column high-performance liquid chromatographic (LC) methods are used. These TLC procedures were not found to be appropriate for the satisfactory separation and quantification of all the tested bioactive compounds. Further, HPTLC also has an advantage of using large number of samples with low cost and high accuracy and precision as compared to liquid chromatographic methods.

Earlier, withanolide was isolated and purified from roots of *Withaniasomnifera* roots by using HPTLC, HPLC and column chromatography [20]. Earlier direct and rapid protocol for separation identification, and quantification of selected withanolides in plant extracts was developed by However, there was no method to determine simultaneous separation of Withaferin A, Withanolide A and Withanolide B on plant part basis. Hence, the present study is undertaken to develop an easy and effective method for the simultaneous separation.As this research paper is part of larger project, our continuous interest in developing easy, affordable, rapid and precise method for analysis of medicinal plants [21-24] has led to successfully develop accurate HPTLC method for the simultaneously determination of three withanolodes in different plant parts of *Withaniasomnifera* as discussed in this research paper.

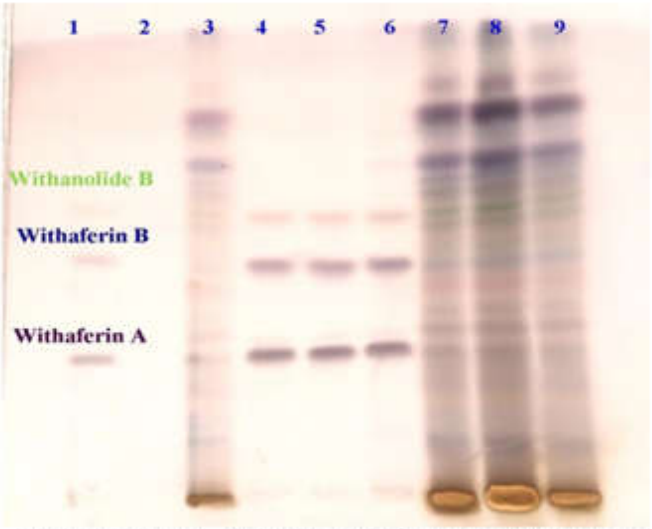
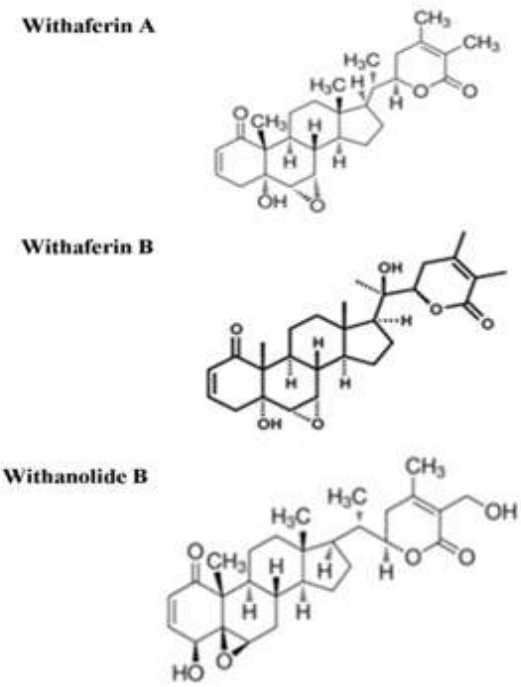


Figure 1: Track 1. Standards, Withaferin A; Withaferin B; Withanolide B; Track 2. Blank, Track 3. Stem sample; Track 4-6. Standards; Track 7. Fruit sample; Track 8. Leaf sample; Track 9. Root sample.



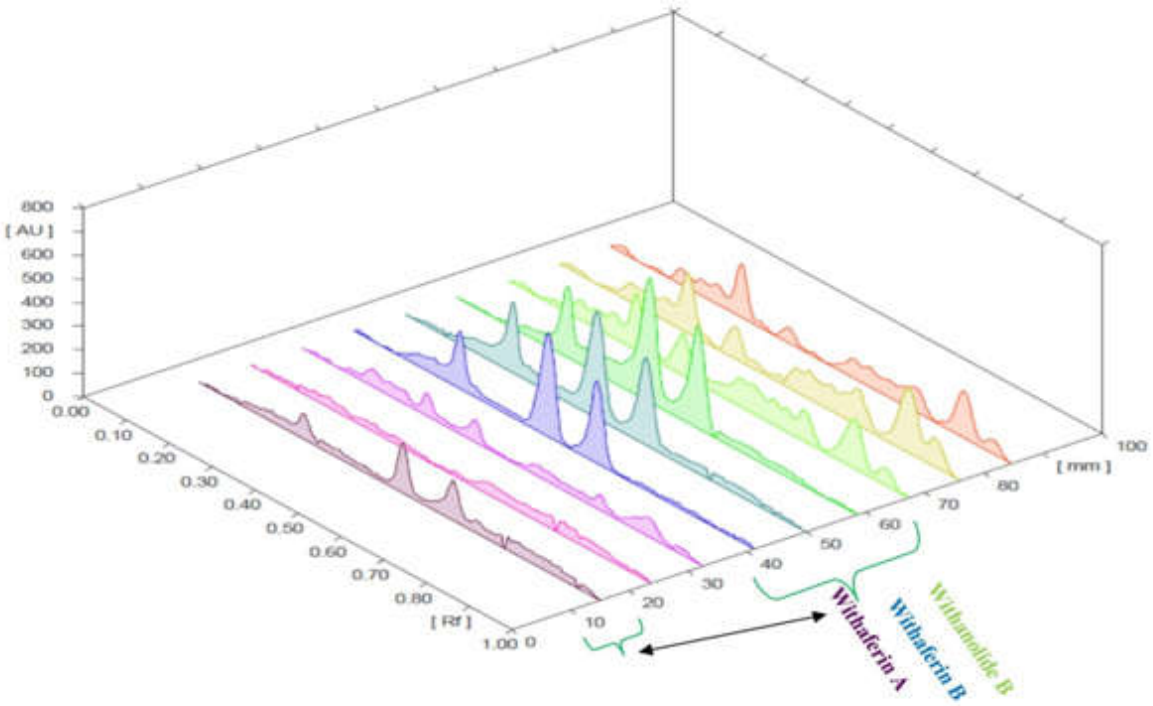


Figure 2: Track 1. Standards, Withaferin A; Withaferin B; Withanolide B; Track 2. Blank, Track 3. Stem sample; Track 4-6. Standards;Withaferin A; Withaferin B; Withanolide B; Track 7. Fruit sample; Track 8. Leaf sample; Track 9. Root sample.

Figure 1 & 2:

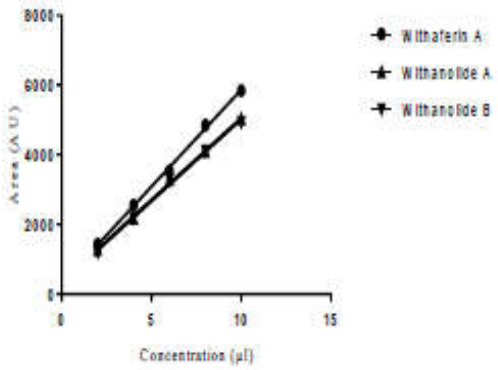


Figure 3: Showing linear regression curves of withaferin A, withanolide A and withanolide B.

Every plant or plant part has a specific phytochemical profile which can be used for the specific purpose of selection. HPTLC is a unique technique which allows us to simultaneously identify different compounds in the same plant or the specific compounds in different plants in a single attempt. HPTLC produces visible chromatograms complex information about the entire sample is available at a glance. Multiple samples are seen simultaneously so that reference and test samples can be compared for identification. Similarities and differences are immediately apparent and with the help of the image comparison. Several chromatograms can be compared directly, even from different plates.

Cancer, being the second leading cause of mortality, exists as a formidable health challenge. Withaferin-A, sensitizes resistant cancer cells to existing chemotherapeutic agents.The antioxidant activity of withaferin-A was first reported by Bhattacharya [26]. Previously, novel HPTLC method was developed for the estimation of withaferin A in for *Withaniasomnifera* [27].

CONCLUSION

The employed statistical analysis ensures that the developed method is reproducible and selective. HPTLC densitometry is a rapid, easy, reproducible, accurate and selective alternative as compared to other chromatographic methods as HPLC for the separation of the and withaferin-A, withanolide-A and withanolide-B in *Withaniasomnifera*. The results of the present study conclude that leaves are supplemented with the desired amount composition of bioactive compounds and can be used in polyherbal formulations. Only leaves can be used instead of other plant parts like roots thereby saving the flora. The employed statistical analysis ensures that the developed method is reproducible and selective. This method can be used as an important tool to ensure the therapeutic dose in herbal formulations, standardization and quality control of bulk drugs. This method can be used as an important tool to ensure the therapeutic dose in herbal formulations, standardization and quality control of bulk drugs. Present study gives scope as an alternative source for the preparation of pharmaceutical medicines.

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Conflict of interest statement
Authors declare that we have no conflict of interest.

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