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Development of hptlc fingerprint by simultaneous estimation of ashwagandha (withaferin-A) and glycyrrhiza(18-β-glycyrrhetinic acid) in some polyherbal over the counter marketed formulations

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ABSTRACT

A new simple, precise, rapid and selective high-performance thin-layer chromatographic (HPTLC) method has been developed and validated for the simultaneous determination of withaferin A and 18-β-Glycyrrhetinic acid in Ayurvedic formulations containing Ashwagandha and Glycyrrhiza. The retention factors of withaferin A and 18-β-Glycyrrhetinic acid were 0.27 ±0.02 and 0.72 ± 0.0 1 respectively. Linearity was obtained in the range of 1-5 µg mL-1 for withaferin A and 18-β-Glycyrrhetinic acid. The developed and validated HPTLC method was employed for standardization of three Ayurvedic formulations for their content of the two markers. Satisfactory recoveries of 98.39 to 99.77% 98.46 to 99.60% were obtained for withaferin A and 18-β-Glycyrrhetinic acid. The results obtained in validation assays indicate the accuracy and reliability of the developed simultaneous HPTLC method for the quantification of withaferin A and 18-β-Glycyrrhetinic acid in all three Ayurvedic formulations containing Ashwagandha and Glycyrrhiza.

Keywords: Ashwagandha, Withaferin A, 18-β-Glycyrrhetinic acid, Glycyrrhiza, validation

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INTRODUCTION

Numerous ayurvedic formulations contain Ashwagandha (withaferin A) and Glycyrrhiza (18-β-Glycyrrhetinic acid) together in combinations. *Withania somnifera* Dual belonging to family Solanaceae, commonly known as Ashwagandha is one of the most valuable, widely used medicinal herb. Roots and leaves of this plant are proven to have a wide range of pharmacological activity. Ashwagandha is considered as health care food supplement. A number of market formulations containing Ashwagandha are available in the market. It is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g., arthritis, rheumatism), and as a general tonic to increase energy, improve overall health and longevity, and prevent disease in athletes, the elderly, and during pregnancy. [1, 2] Many pharmacological studies have been conducted to investigate the properties of ashwagandha in an attempt to authenticate its use as a multi-purpose medicinal agent. Several types of alkaloids, withanolides, glycosides, glucose and free amino acids are the major chemical constituents of this plant Wide variation is reported in the type and amount of chemical constituents present in different samples[3]. Withaferin A is commonly used as a marker compound to evaluate and standardize Ashwagandha [4].

Glycyrrhiza glabra (Licorice) belonging to family Leguminoseae is widely used as a medicinal herb in different parts of world. licorice has shown anti-inflammatory, antiarthritic, anti-arrhythmic, anti-bacterial, antiviral and

expectorant activity. A recent animal study indicates that licorice may be useful in treating lupus [5]. The principal constituent of liquorice to which it owes its characteristic sweet taste is glycyrrhizin, besides glycyrrhizinic acid [6] a glycoside which on hydrolysis yields glycyrrhetinic acid. It is now known that glycyrrhizic acid and its aglycone glycyrrhetinic acid present in the root extract are responsible for these biological activities . It is determined quantitavely for standardization of the licorice products[7-9]. It is used extensively in the tobacco, food, confectionery, and pharmaceutical industry, throughout the world [10].

In Indian market there are many products containing Ashwagandha and Glycyrrhiza with different therapeutic benefits. The products are available in form of traditional Ayurvedic preparations as well as modern formulations. For Past few decades compounds from natural sources have been gaining importance because of the vast chemical diversity that they offer. This has led to phenomenal increase in the demand for herbal medicines in the last two decades and a need has been felt for ensuring the quality, safety and efficacy of herbal drugs. Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening, chemo profiling and marker compound analysis using modern analytical techniques. In the last two decades (HPTLC) has emerged as an important tool for the qualitative semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers. This is especially important for those species that contain different active constituents. World Health Organization (WHO) encourages, recommends and promotes traditional herbal remedies in national health care programmes because these drugs are easily available at low cost, safe and people have faith in them. The WHO assembly in number of resolutions has emphasized the need to ensure quality control of medicinal plant products by using modern techniques and applying suitable standards.[11-15] The objective of the present work was to develop and validate a High Performance Thin Layer Chromatography method for simultaneous estimation of two biomarkers withaferin A and 18-β-Glycyrrhetinic acid present in polyherbal formulations containing Ashwagandha and Glycyrrhiza. The method was validated in compliance with ICH guidelines [16].

MATERIALS AND METHODS

Withaferin A and 18-β-Glycyrrhetinic acid standards were procured from Natural Remedies, Bangalore. Silica Gel 60 F254 Aluminium plates (Merck) was used as stationary phase. Toluene: Ethyl acetate: glacial acetic acid: Chloroform (5:5:1:2 v/v/v) was used as mobile phase. Methanol was used as solvent. Selected marketed polyherbal formulations Brento tablets, Brento Liquid and Mannoll capsule were obtained from local pharmacy stores. Ashwagandha and Glycyrrhiza samples were obtained from reliable vendors of herbs. The samples were authenticated by Archana Khemani, Head, Department of Botany, Zandu Pharmaceuticals Pvt. Ltd. (now Emami, the herbarium of the specimens has been deposited at Botany Department of Zandu Pharmaceuticals Pvt Ltd (now Emami), Vapi, Gujarat.

All chemicals and reagents used were of analytical grade and purchased from Rankem and S. D. Fine Chemicals, India.

A Camag HPTLC system (Switzerland) comprising of Camag Linomat IV applicator, Camag TLC Scanner 3, Camag winCATS software, version 1.3.3, Hamilton syringe (100µl), Camag, Shimadzu weighing balance, Dip tank, Camag UV cabinet were used for the study

Preparation of standard solution:
2.5mg of Withaferin A was dissolved in 5 ml methanol to give a concentration of 0.5µg/µl.
25 mg of 18 β Glycyrrhetinic was dissolved in 50 ml chloroform to give a concentration of 0.5µg/µl.

Preparation of samples of marketed formulations:
Three different dosage forms were chosen for estimation of withaferin A and 18-β-Glycyrrhetinic acid. Of these one is tablet (Brento Tablet) other is capsule (Mannoll capsule) and third is liquid (Brento liquid) formulation.

Procedure for sample preparation to estimate withaferin-A (Ashwagandha)
100g of defatted powdered materials of Ashwagandha sample was refluxed with methanol for 12 hrs. The extract was concentrated by distilling the solvent at low temperature and air dried. Extracts obtained were stored in air-dried containers, labelled and used for HPTLC analysis. 100mg of Ashwagandha sample extracts was dissolved in 10ml of chloroform, filtered through Whatmann No 1 filter paper.

Procedure for sample preparation to estimate withaferin-A (Tablet)
Coated Brento tablets were soaked in water to remove coating and dried in oven at 105^{o}C. Five tablets were weighed, powdered and macerated with 30ml methanol for 24 hrs. This was then filtered through Whatmann No.1 filter paper.

Procedure for sample preparation to estimate withaferin-A (Capsule)
Hard gelatin shells were removed, five capsules were emptied and the powder within was weighed. This was dissolved and macerated in 30ml methanol for 24 hrs. This was then filtered through Whatmann No.1 filter paper.

Procedure for sample preparation to estimate withaferin-A (Liquid)
20 ml of liquid was refluxed with equal quantity 20 ml each of methanol and water for 1 hour. It was filtered through Whatmann No.1 filter paper and extracted with 10 ml chloroform twice and concentrated to 10 ml.

Procedure for sample preparation to estimate 18 β Glycyrrhetinic acid (Glycyrrhiza)
100g of defatted powdered materials of Glycyrrhiza sample was refluxed with methanol for 12 hrs. This was then filtered through Whatmann No.1 filter paper, refluxed for 1 hour with 50 ml 5N hydrochloric acid. Cooled , filtered and filtrate was treated with 20 ml chloroform thrice. Chloroform extracts were collected and evaporated.The extracts were concentrated by distilling the solvent at low temperature and air dried. Extracts obtained were stored in air-dried containers, labelled and used for HPTLC analysis. 100mg of Glycyrrhiza sample extracts was dissolved in 10ml of chloroform, filtered through Whatmann No 1 filter paper.

Procedure for sample preparation to estimate 18 β Glycyrrhetinic acid (Tablet)
Coated Brento tablets were soaked in water to remove coating and dried in oven at 105^{o}C. Five tablets each were weighed, powdered and macerated with 30ml methanol for 24 hrs. This was then filtered through Whatmann No.1 filter paper, refluxed for 1 hour with 20 ml 5N hydrochloric acid. Cooled , filtered and filtrate was treated with 20 ml chloroform twice. Chloroform extracts were collected and evaporated to volume of 10 ml.

Procedure for sample preparation to estimate 18 β Glycyrrhetinic acid (Capsule)
Five capsules were weighed, powdered and macerated with 30ml methanol for 24 hrs. This was then filtered through Whatmann No.1 filter paper, refluxed for 1 hour with 20 ml 5N hydrochloric acid. Cooled, filtered and filtrate was treated with 20 ml chloroform twice. Chloroform extracts were collected and evaporated to volume of 10 ml.

Procedure for sample preparation to estimate 18 β Glycyrrhetinic acid (Liquid) 20 ml Liquid macerated with mixture of 20ml methanol and 20 ml water for 24 hrs. This was refluxed with 20 ml 5 N HCl for 1 hour then filtered through Whatmann No.1 filter paper. Cooled, filtrate was treated with 20 ml chloroform twice. Chloroform extracts were collected and evaporated to volume of 10 ml..

Chromatographic conditions
The chromatographic estimation was performed using the following conditions, stationary phase was precoated silica gel 60 F254 aluminium sheets (20 x 10 cm) and the mobile phase used was Toluene: Ethyl acetate: glacial acetic acid: Chloroform (5:5:1:2 v/v/v). The chamber saturation time employed was 20 minutes and the developing distance was 8.5cm. Scanning wavelength of 223 and 254 nm for withaferin A and 18-β-Glycyrrhetinic acid with a slit dimension of 8.0 x 0.40 mm, scanning speed of 20 mm/s and data resolution of 100 µm/step were employed.
.

Method Validation:
The method was validated in compliance with ICH guidelines The following parameters were validated

Linearity
The linearity of responses for withaferin-A and 18 β Glycyrrhetinic was assessed in the range of 1-5µg/spot. Five different concentrations of the standards solutions were applied three times to study the linearity.

Accuracy and recovery studies
Both accuracy and recovery were studied. Accuracy of the method was tested by taking three concentrations and three determinations of each analytical concentration. The recovery study was carried out by addition of known amounts of standards to the product. Standards added were 80%, 100% and 120% of 1 µg of Withaferin-A and 18 β Glycyrrhetinic respectively. Three determinations were done to study the recovery. The % recovery of Withaferin-A

and 18 β Glycyrrhetinic was compared with the actual amounts. Areas of sample, standard and spiked samples were recorded and % recovery was calculated using the following formula.

$$\%\text{Recovery} = \frac{A+B}{C} \times 100$$

Where. Area of sample = A
Area of standard = B
Area of sample + Area of standard (spiked) = C
Results of accuracy are shown in Table 20.

Precision
System precision
The system precision study basically focuses on the exactness of the instrument.

Repeatability of sample application and measurement of the peak area were studied. Six determinations at a concentration of 2µg/µl, for Withaferin-A and 18 β Glycyrrhetinic were applied. The repeatability of sample application and the repeatability of measurement of the peak area were evaluated by comparing their coefficient of variations which are obtained from the peak area measurements.

Method precision
To study the precision of the method, both intra - day and inter - day precision were applied. Intra- day precision was studied by taking three different concentrations 1.0, 2.0 and 3.0µg/spot of withaferin-A and 18 β Glycyrrhetinic were applied three times to see variation in their peak areas within a day. For inter-day precision the same concentrations were applied but their peak area variation was studied for three different days.

Limit of detection and quantification
Detection and quantification limits were calculated from the calibration equations obtained from the experiment. The determinations of the detection and quantitation limits were based on the standard deviation of the response and the slope.

The limit of detection and limit of quantification of the proposed method were calculated according to 3.3xS.D/S and 10xS.D/S criterions, respectively, where S is the slope of the corresponding calibration curve and S.D is the standard deviation of the y-intercept (n=3) of the calibration curve
.
Robustness
In order to study the robustness of the method, slight but deliberate changes were made in some parameters. Parameters such as; the mobile phase composition, total mobile phase amount, time from application to development and time from development to scanning were used to study the robustness. Concentrations of 2.0 µg/spot of withaferin-A and 18 β Glycyrrhetinic acid were applied for the analysis.

Estimation of withaferin-A and 18 β Glycyrrhetinic acid
For quantification 50µl of all sample solutions for withaferin-A and 10µl of sample solutions for 18 β Glycyrrhetinic acid were spotted along with standard solution. Ashwagandha and Glycyrrhiza samples were applied just for identification of respective markers in the herbs and were not quantified. The chromatograms were developed and scanned at 223 and 254nm.

The amount of Withaferin-A and 18 β Glycyrrhetinic acid present in market formulations was calculated by comparing the peak area of standard and respective samples.
The following formula was used to quantify the active constituent,

$$\%\ \text{Withaferin-A/18 β Glycyrrhetinic acid} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \frac{\text{conc. of standard}}{\text{conc. of sample}} \times 100$$

The developed chromatogram spotted with market formulations and standards was derivatized with Anisaldehyde Sulphuric acid reagent and documented by capturing its image. The image of the HPTLC pattern and 3D view for linearity and marketed formulations at 223 nm and 254nm is shown in figures 1-5.

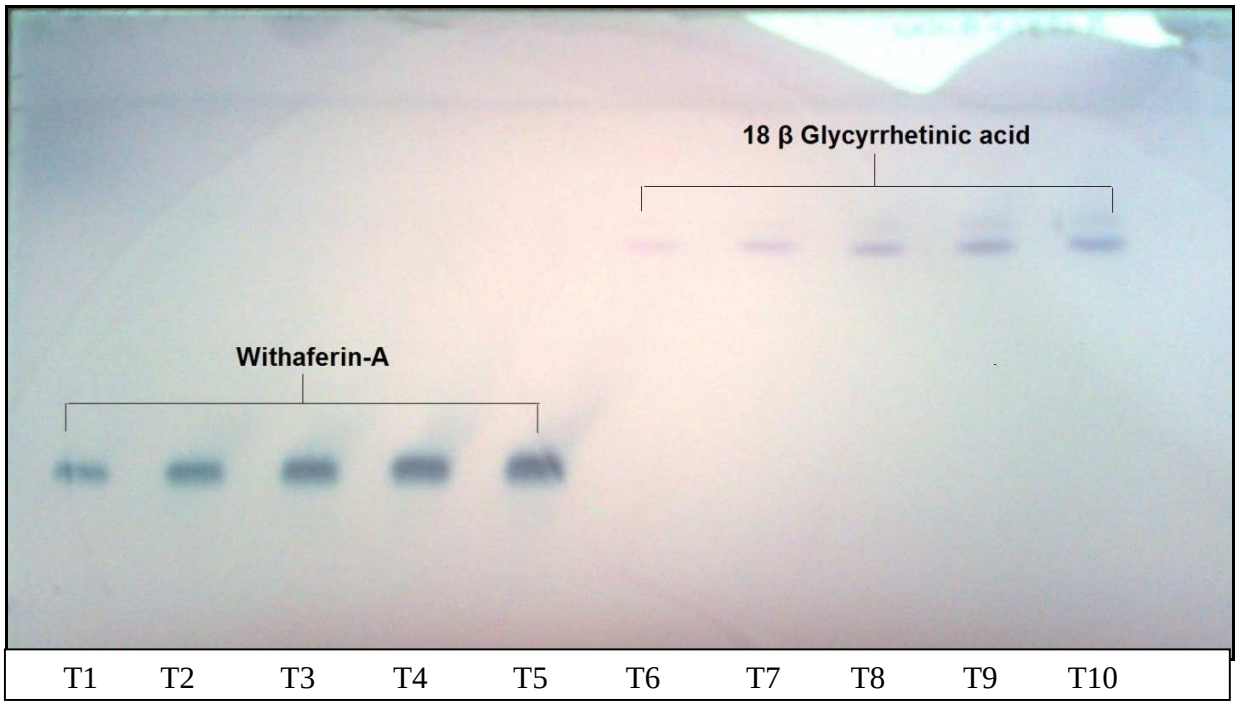

FIGURE NO. 1 DERIVATISED HPTLC PLATE OF WITHAFERIN-A AND 18 β GLYCYRRHETIINIC ACID LINEARITY

T1- Withaferin-A 1.0 µg; T2- Withaferin-A 2.0 µg; T3- Withaferin-A 3.0 µg
T4- Withaferin-A 4.0 µg; T5- Withaferin-A 5.0 µg; T6- 18 β Glycyrrhetinic acid 1.0 µg
T7- 18 β Glycyrrhetinic acid 2.0 µg; T8- 18 β Glycyrrhetinic acid 3.0 µg;
T9- 18 β Glycyrrhetinic acid 4.0 µg; T10- 18 β Glycyrrhetinic acid 5.0 µg

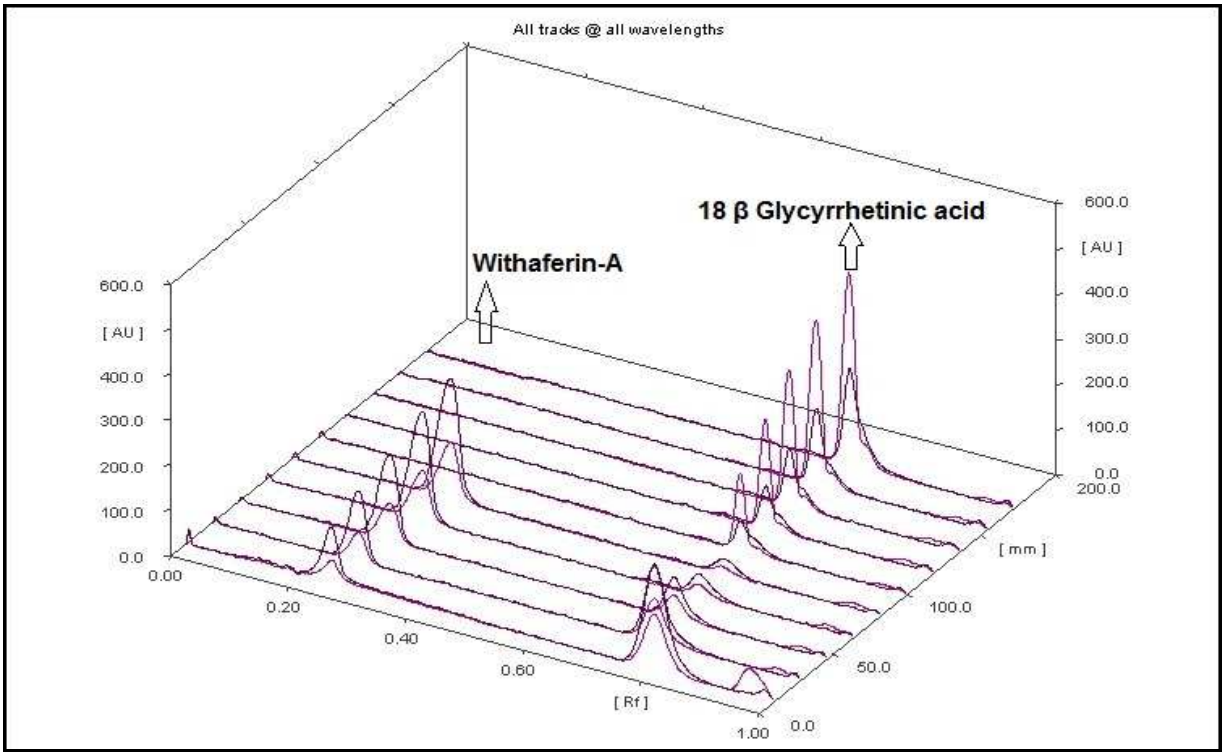

FIGURE NO. 2- 3D VIEW OF AT 223 NM AND 254 NM OF WITHAFERIN-A AND 18β GLYCYRRHETINIC ACID LINEARITY

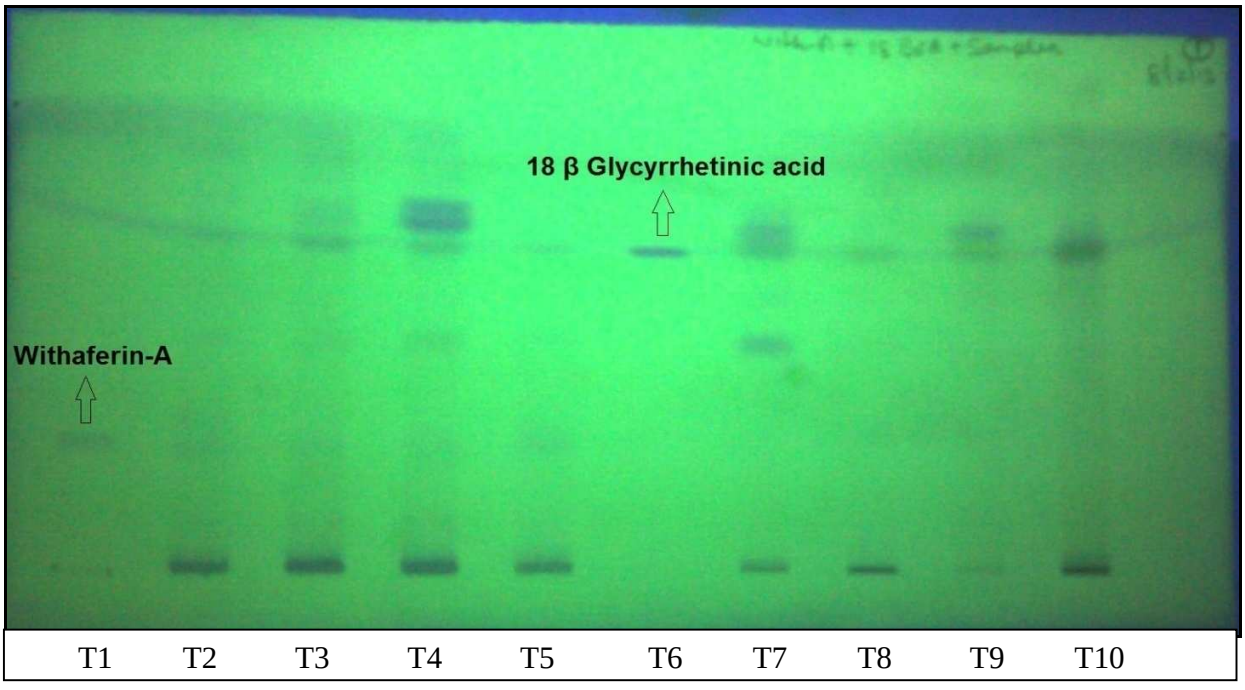

FIGURE NO. 3- HPTLC PATTERN OF ASHWAGANDHA AND GLYCYRRHIZA FORMULATIONS AT 254 NM

T1- Withaferin-A; T2- Brento liquid (Chloroform extract)
T3- Brento tablet (Methanol extract); T4- Manoll capsule (Methanol extract)
T5- Ashwagandha (Methanol extract); T6- 18 β Glycyrrhetinic acid

T7- Brento liquid (Hydrochloric acid hydrolysed chloroform extract)
T8- Brento tablet (Hydrochloric acid hydrolysed chloroform extract)
T9- Manoll capsule (Hydrochloric acid hydrolysed chloroform extract)
T10- Glycyrrhiza (Hydrochloric acid hydrolysed chloroform extract)

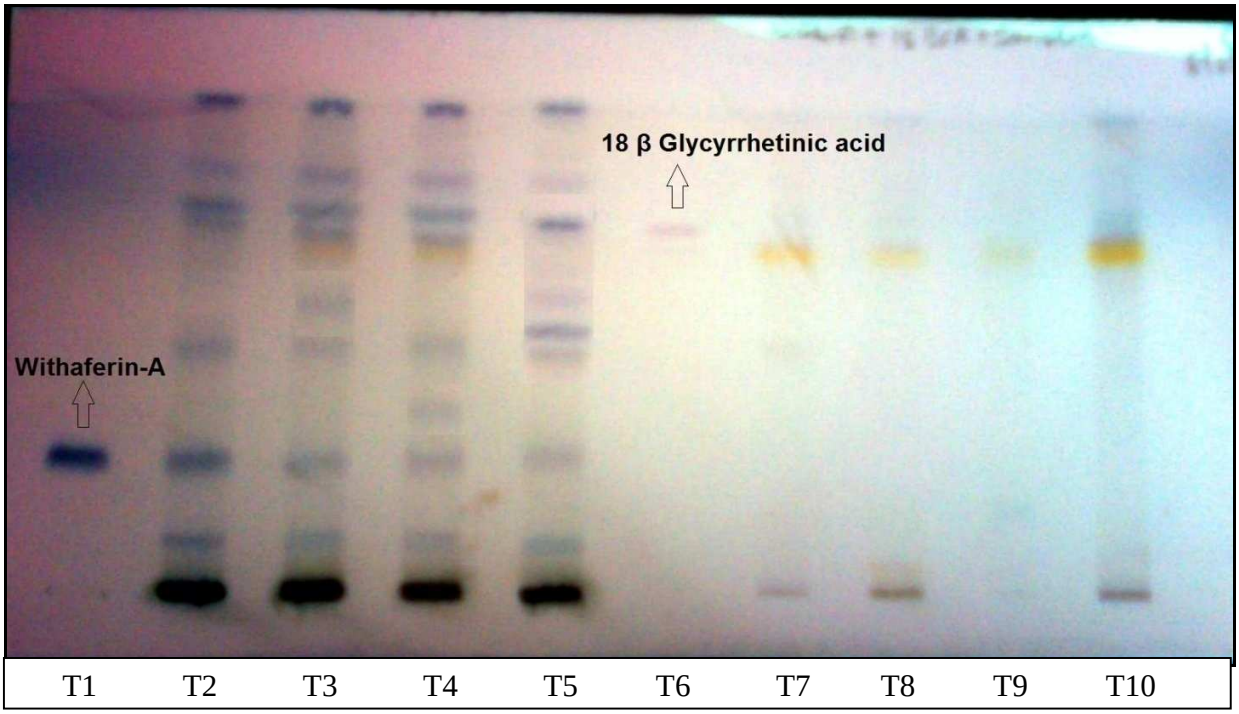

FIGURE NO. 4- DERIVATISED HPTLC PATTERN OF ASHWAGANDHA AND GLYCYRRHIZA FORMULATIONS

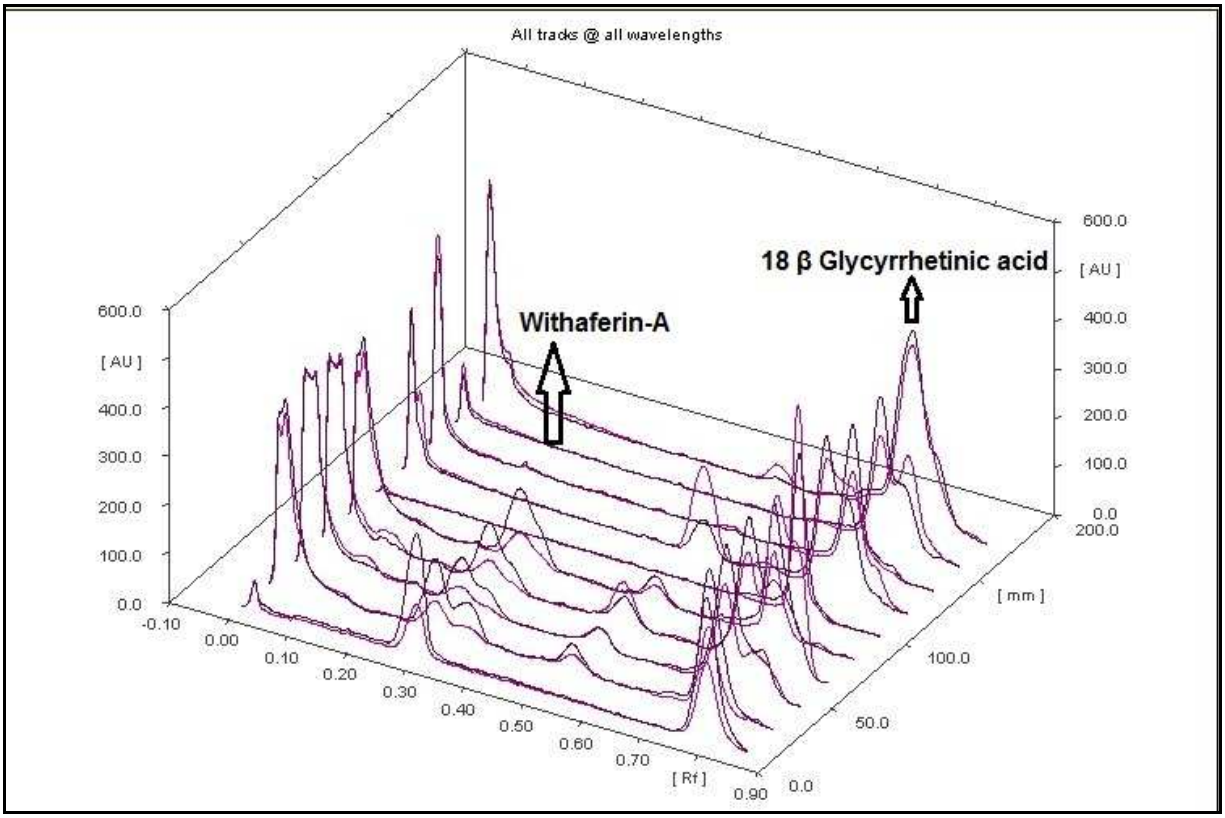

FIGURE NO. 5- 3D VIEW OF AT 223 NM AND 254 NM OF WITHAFERIN-A AND 18 β GLYCYRRHETINIC ACID IN FORMULATIONS

RESULTS AND DISCUSSION

The mobile phase containing Toluene: Ethyl acetate: glacial acetic acid: Chloroform (v/v/v) in the ratio of 5:5:1:2 gave good resolution, sharp and symmetric peaks and better spot characteristics for Withaferin-A and 18 β Glycyrrhetinic acid. The shape of the peaks was not altered by other substances present in the matrix. The spots at Rf values 0.27 ± 0.02 and 0.72 ± 0.01 were identified as Withaferin-A and 18 β Glycyrrhetinic acid respectively with the help of chromatograms of their individual standards.

The method gave a good linearity curve in the range of 1.0 – 5.0 µg for Withaferin-A and 18 β Glycyrrhetinic acid respectively with correlation coefficient of 0.999 ± 0.0001 and 0.996 ± 0.0005 for Withaferin-A and 18 β Glycyrrhetinic acid. The average recovery values of Withaferin-A and 18 β Glycyrrhetinic acid were found in the range 98.39 to 99.77% and 98.46 to 99.60% which are in accordance with ICH limits of 80% - 120%. The LOD of Withaferin-A and 18 β Glycyrrhetinic acid were found to be 0.037 µg/spot and 0.090µg/spot, respectively. These were the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The LOQ of Withaferin-A and 18 β Glycyrrhetinic acid were 0.112µg/spot and 0.273µg/spot, respectively. These were the lowest concentration of drugs, accurately detected and integrated by the instrument. The low values of S.D. and % RSD along with unchanged R_f values of Withaferin-A and 18 β Glycyrrhetinic acid obtained after introducing small deliberate changes in the method indicated the robustness of the developed HPTLC method. Different validation parameters of the proposed HPTLC method are summarized in table no. 1

TABLE NO. 1- SUMMARY OF VALIDATION PARAMETERS FOR SIMULTANEOUS ESTIMATION OF WITHAFERIN-A AND 18 β GLYCYRRHETINIC ACID BY HPTLC METHOD

Parameter	Withaferin-A	18 β Glycyrrhetinic acid
Λ max (nm)	223	254
Linearity range (µg/spot)	1-5	1-5
Correlation Coefficient	0.999 ± 0.0001	0.996 ± 0.0005
Regression equation	y = 1652.12 x + 804.91	y = 1531.9 x + 1075.6
Limit of detection (µg/spot)	0.037	0.090
Limit of Quantification (µg/spot)	0.112	0.273
Recovery (Mean ±S.D.)	99.14 ± 0.465	99.24 ± 0.314
Precision (% RSD)		
Repeatability of application (n=6)	0.339	0.733
Repeatability of measurement (n=6)	0.414	0.717
Intra-day*	0.435	0.449
Inter-day**	0.409	0.381
Robustness	Robust	Robust

** Mean of three concentrations in triplicates in the same day.*
*** Mean of three concentrations in triplicates in three different days.*

The proposed method was applied for the determination in commercial polyherbal formulations containing Ashwagandha and Glycyrrhiza. Three replicates of determinations were made and satisfactory results were obtained with good separation. Hence the method was suitable for routine analysis of Ashwagandha and Glycyrrhiza containing formulations. The amounts of Withaferin-A and 18 β Glycyrrhetinic acid obtained in different dosage forms are reported in the table no. 2. and in figure6.

TABLE NO. 2- PERCENT CONTENT IN MARKETED FORMULATIONS

Sample No.	Sample name	% Withaferin-A ± S.D	%18-βGlycyrrhetinic Acid ± S.D
1	Brento Liquid	0.393 ± 0.013	2.11 ± 0.134
2	Brento Tablet	0.478 ± 0.010	2.33 ± 0.106
3	Mannoll Capsule	0.545 ± 0.025	2.42 ± 0.115

a) Mean ±S.D., n=3

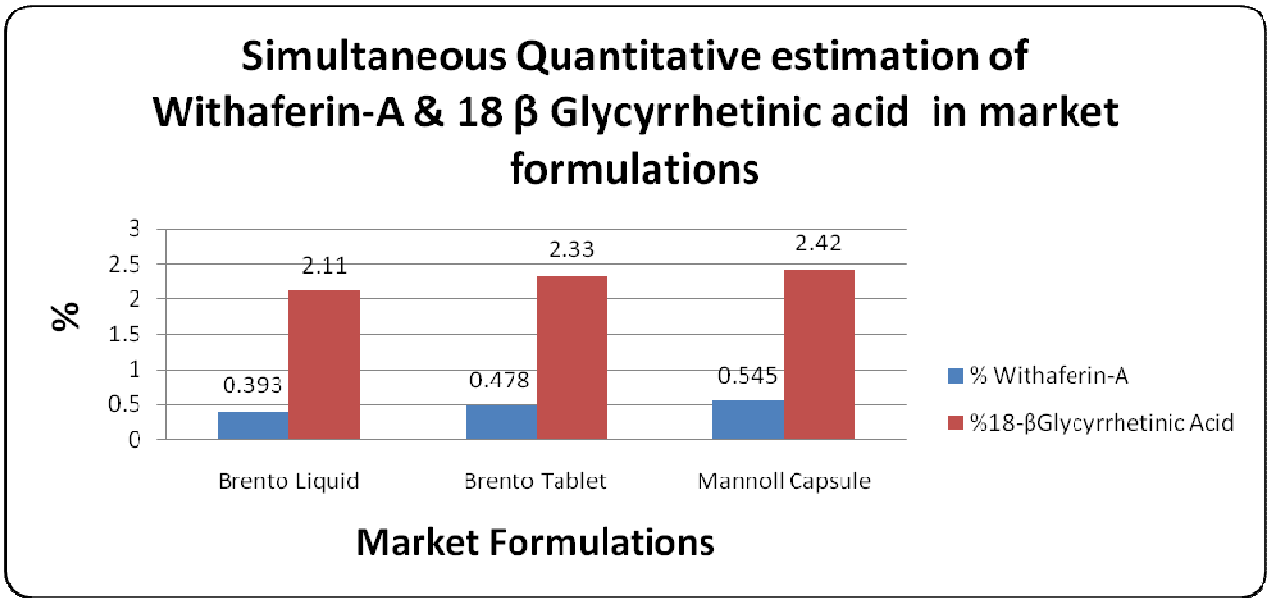

FIGURE NO. 8 QUANTITATIVE ESTIMATION OF WITHAFERIN-A AND 18 β GLYCYRRHETINIC ACID IN MARKETED FORMULATIONS.

CONCLUSION

A simple, rapid, accurate and convenient method was developed for estimation of Withaferin-A and 18 β Glycyrrhetinic acid by HPTLC. The statistical analysis indicates that the method is repeatable, precise and selective. This method can be adopted for quality control of Ayurvedic polyherbal formulations containing Ashwagandha and Glycyrrhiza on routine basis.

These analytical standardization technique facilitate manufacturers to market their plant based medicines with defined content of respective bioactives and to ensure its quality.

Amount of active constituent present in one human dose also shows wide variation that may be correlated to the quality of the drug. The difference of % may be due to varied factors like drug variety, geographical variation, and age of the plant at the time of harvest, genetic and environmental factors. This method can be applied by herbal manufacturers to estimate the presence and amount of above marker in their formulations as a routine quality control check, as well as to monitor batch to batch variation. It can also be used to identitfy adulteration by finding out presence and amount of markers in crude drugs prior to their use in manufacturing different dosage forms.

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