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Fingerprinting And Stability Studies of Triphala-Guggula Vati: An Official Ayurvedic Formulation

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ABSTRACT

Triphala-guggula vati a polyherbal Ayurvedic formulation is recommended in the management of pain in renal stone, it has dual action of aiding digestion and lowering body weight. It is effective in lowering cholesterol and reducing fat. It is also use in treatment of anal fistula, fissures and severe constipation. There has been an increase in demand for the Phyto-pharmaceutical products of *Ayurveda* so a pharmaceutical preparation in the form of Triphala-guggula vati was tried to standardize in terms of Fingerprinting and Stability studies, which is economical in terms of time and machinery usage. Literature survey indicates that no Fingerprinting and stability studies were carried out; hence present study was undertaken for the same. UV and HPTLC studies were carried out for finger printing of all important phytoconstituents of Triphala-guggula vati and its raw ingredients. HPTLC study were carried out on Aqueous and Alcoholic extracts using Camag HPTLC system equipped with Linomat V applicator, Camag TLC scanner 3 and CATS 4 software for interpretation of data. The laboratory batch and two Marketed preparations of Triphala-guggulu vati were subjected for development of rapid, reproducible, valid Fingerprinting method and stability testing method at standard recommended accelerated condition.

Keywords: Official Ayurvedic formulation, Triphala-Guggula Vati, Fingerprinting, Stability studies.

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INTRODUCTION

Natural products have served mankind as a source of medicine since — and even before — historical records began. Herbal extracts now play an important and growing role in disease prevention and therapy and are used extensively as drugs and food additives[1]. Despite the use of several herbal medicines over centuries, only a relatively small number of plant species has been scientifically validated. The quality and quantity of safety and efficacy data on traditional medicines are far from sufficient to meet the criteria needed to support their use worldwide. The main reason for the lack of such data may be due to lack of adequate or accepted research methodology for evaluating traditional medicines[2]. Quality control is a term that refers to processes involved in maintaining the quality and validity of a manufactured product. Quality is the critical determinant of safety and efficacy of botanical medicines, however, botanical preparation rarely meet the standards of quality, which refers to procedures and markers for assessing and verifying the strength of botanical raw materials or extracts or formulation thereof. Methods currently utilized in evaluating herbs, their extracts and formulations include Organoleptic or Morphological, Microscopic, Physical, Chemical, Chromatographic Fingerprinting, Microbiological and Biological methods[3]. A lot of analytical techniques have been developed for quality control of drugs from plant origin. One quality control mode is done by selecting known active constituents or a marker compounds as the qualitative and quantitative target to assess its authenticity and inherent quality. Chromatographic fingerprinting emphasizes an integral formulation of pharmacologically active phytopharmaceutically characteristic components of samples with similar or different attributions. This technique can be used for the assessment of quality consistency and stability of herbal extracts or products by visible observations and comparison of the standardized fingerprint pattern[4]. Fingerprinting of the herbals are done by HPLC (High performance Liquid Chromatography), HPTLC (High Performance Thin Layer Chromatography), MS, LC-MS, H-NMR, etc.

The purpose of stability study was to provide evidence on the quality of drug substances or drug products varies with time, under the influence of a variety of environmental factor such as temperature, humidity and light recommended for storage condition. The accelerated stability testing is being carried out at higher temperatures for assigning shelf life of a drug. At such higher temperature and humidity, drug decomposition is more rapid than at room temperature. Thus in a relatively short period of time much information is obtained about the stability of a drug product[5].

MATERIALS AND METHOD

Plant material

Raw material i.e. *Terminalia chebula, Terminalia belerica, Emblica officinalis* , *Piper longum* and *Commiphora wightii* were collected from Botanical Garden of Nagpur, Maharashtra and was authenticated by Dr. Mrs. Chaturvedi, Professor and Head of Department, Department of Botany, Post Graduate Teaching Department, RASHTRA SANT TUKDOJI MAHARAJ NAGPUR UNIVERSITY, NAGPUR. Herbarium is deposited of *Terminalia chebula* -9896, *Terminalia belerica* -9897, *Emblica officinalis*-9898, *Piper longum*-9899 and *Commiphora wightii*-9902 in the Department.

Formulation profile

Formulation 1(Marketed): It is in powdered form, purchased from local market in Nagpur. Coded as **TGVM-I**.

Formulation 2 (Marketed): It is in powdered form, purchased from local market in Nagpur. Coded as **TGVM-II**.

Formulation 3 (Laboratory): It is in powdered form, prepared in Laboratory of Natural Product, Sudhakarrao Naik, Institute of Pharmacy, Pusad, and Coded as TGVL.

Take all the ingredients of the Pharmacopoeial quality. Wash, dry and powder the ingredients as per Table 1 number 1 to 4 of the formulation composition separately and pass through sieve number 85, weigh them separately in the required quantities and mix. Crush weighed quantity of ingredient number 5 i.e. Guggulu-suddha, add fine powder of other mixed ingredients to it and pound well. Add Ghrita to an extent required to facilitate the pounding and continue pounding till a semi-solid uniformly mixed mass of suitable plasticity is obtained. Expel the mass through Vati machine fitted with a suitable die and cut the Vatis to a desired weight. Roll the Vatis on flat surface to round them by circular motion of palm covered with a glove and smeared with Ghrita or use suitable mechanical device. Dry the rounded Vatis in a tray-dryer at a temperature not exceeding 600 for 8 to 10 h. The method of preparation of Triphala-guggula vati was as per The Ayurvedic Formulary of India[6].

Table 1: Formulation of Triphala-Guggula Vati.

Sr.No.	Sanskrit name/ Scientific Name	Part used	Quantity
1	Haritaki / *Terminalia chebula*	Pericarp	48g
2	Bhibitaki / *Terminalia belerica*	Pericarp	48g
3	Amalaki / *Emblica officinalis*	Pericarp	48g
4	Pippali / *Piper longum*	Fruit	48g
5	Guggulu-suddha / *Commiphora wightii*	Oleo resin	240g

Methods

The fingerprint development was done in two phases, in phase one, development of method for selected marker and in phase two estimation of marker was done in formulation by the developed method. Triphala Guggulu vati is well known herbal formulation described in classical text and in Ayurvedic Formulary of India. Selective and efficient analytical methods are required for quality assurance and authentication of herbal formulations. A simple, valid and rapid UV and HPTLC fingerprint method has been developed for estimation of Tannic acid, gallic acid, Piperine and Z and E-guggulosterone in laboratory formulation, marketed preparations and individual ingredients as applicable used for preparation of selected formulation of Triphala-Guggulu Vati.

Development of UV spectroscopy fingerprint

The UV spectroscopy fingerprint method was developed for each batch of Triphala-Guggulu Vati selected formulations and raw ingredients mainly *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis* for Tannic acid content which is an important content in Triphala-Guggulu Vati along with development of UV spectroscopy method for determination of Piperine content in Triphala-Guggulu Vati and *piper longum* by using following experimental techniques.

Development of UV spectroscopic method for Tannic acid

All the chemicals used for the development of fingerprint method were of A.R. grade which were purchased from Sigma-Aldrich, USA through Sigma-Aldrich, Bangalore, India. Triphala-Guggulu Vati was estimated for their tannic acid contents against the standard tannic acid solution on UV spectrophotometer, Shimadzu, UV-1700. The powdered Triphala-Guggulu Vati about 1gm was extracted with 6 volumes of denatured spirit on a shaker for 2 hours. The extract was filter and the marc left was re-extracted with 4 volumes of denatured spirit for another 1 hour. Filter and combined the filtrates. Concentrate the denatured spirit extract under vacuum till the semi-solid mass is obtained. Dilute with distilled water (1:50) and keep it overnight at 5^0C. Now the extract was filtered and the flocculent precipitate was discarded. The filter was extracted with equal volume of ethyl acetate thrice. Concentrate the ethyl acetate extract till the semi-solid mass is obtained. The residue was dissolved in 75 ml 0.1 N hydrochloric acid and filtered through sintered glass filter funnel (G-2) by vacuum filtration assembly. The filtrate was centrifuged at 2000 rpm for 20 minutes, supernant was collected in 100 ml volumetric flask and volume was made with 0.1 N hydrochloric acid. Same procedure was applied for all the formulations of Triphala-Guggulu Vati and raw ingredients mainly *Terminalia chebula, Terminalia belerica* and *Emblica officinalis*. The standard solution of Tannic acid was prepared by accurately weighing 100 mg Tannic acid and was dissolved in 0.1 N hydrochloric acid and volume was made up to 100 ml by using 0.1 N hydrochloric acid in volumetric flask. 2.0 ml of this solution was diluted with 0.1 N Hydrochloric acid up to 100 ml in 100 ml volumetric flask, so as to get 20µg/ml tannic acid concentration. A series of calibrated 10 ml volumetric flask were taken and appropriate aliquots of the working standards solution of Tannic acid were withdrawn and diluted up to 10 ml with 0.1 N Hydrochloric acid.

Development of UV spectroscopic method for Piperine

The UV spectroscopic fingerprinting method was developed for one laboratory batch, two marketed preparation and *Piper longum* used for preparation of Triphala-Guggulu Vati for estimation of Piperine which is important content of Triphala-Guggulu Vati by using following technique.

All the chemicals and solvents were used of A.R. grade, standard piperine was purchased from Sigma-Aldrich, USA through Sigma-Aldrich Corporation, Bangalore, India. Triphala-Guggulu Vati and its ingredients were subjected for their piperine content against standard piperine solution on UV spectrophotometer, Shimadzu, UV-1700. The powder Triphala-Guggulu Vati about 1gm was refluxed with 60 ml ethanol for 1 hour. The extract was filtered and the marc left was refluxed with 40 ml of ethanol for another 1 hour. Both filtrates were combined and concentrated under rotary vacuum evaporator until semi-solid mass was obtained. The residue was dissolved in 75 ml ethanol and filter through sintered glass funnel (G-2) by vacuum filtration assembly. The filtrate was centrifuged at 2000 rpm for 20 minutes. The supernant was collected in 100 ml volumetric flask and volume was made with ethanol. Same procedure was repeated for laboratory, Marketed preparation and separately powdered *Piper longum* for piperine from their extract.

The standard solution of Piperine was prepared by weighing 100 mg of standard piperine and was dissolved in ethanol and volume was make up to 100 ml in volumetric flask, 2 ml of it was withdrawn and was diluted up to 100 ml with ethanol so as to give 20 µg/ml piperine concentrations. A series of calibrated 10 ml volumetric flask were taken and appropriate aliquots of the working standard solution of piperine were withdrawn and diluted up to 10 ml with ethanol. The absorbance was measured at absorption maxima at 342.8 nm against the reagent blank prepared in similar manner without piperine

Development of HPTLC fingerprints method.

The HPTLC fingerprinting method was developed for laboratory and marketed formulations of Triphala–Guggulu Vati and its raw material mainly *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis* and *Piper longum* for estimation of Gallic acid, Piperine and *Commiphora wightii* for guggulosterone.

Development of HPTLC method for Gallic acid in Triphala-Guggulu Vati and ingredients.

The HPTLC fingerprint method was developed for Gallic acid in Laboratory formulation, marketed preparation of Triphala-Guggulu Vati and *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis*. All the chemicals and solvents were of A.R. grade, Standard Gallic acid was procured from Sigma-Aldrich, USA through Sigma-Aldrich Corporation, Bangalore, India. The Gallic acid extract of Triphala-Guggulu Vati was obtained by refluxing 1 gm of powdered drug with 60 ml methanol for 1hour. Resulting extract was filtered and the marc was refluxed with 40 ml methanol for another 1 hour. The extracts were combined and concentrated under vacuum rotary evaporator until a semi-solid mass is obtained. The residue was dissolved in 100 ml methanol and filtered through sintered glass funnel (G-2) by vacuum filtration assembly. The same procedure was followed for each batch of Triphala-Guggulu Vati and raw ingredients mainly *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis*. The instrumentation and chromatographic conditions were

Spotting device:	Linomat V semi sample spotter; Camag
Syringe:	100 µL Hamilton
TLC chamber:	Glass twin through chamber (20x10x4 cm); Camag
Densitometer:	TLC scanner 3 linked to win cats software V.4.06; Camag
HPTLC plates:	10 x10 cm, 0.2 mm thickness Precoated with silica gel 60
Wavelength:	280 nm
Solvent system:	Toluene; ethyl acetate: formic acid (3:1.6:0.4).
Temperature:	25^0C

The TLC plates were pre-washed with methanol and activated at 115^0C for about 30 minutes. The gallic acid was well resolved on the Pre-coated silica gel g60 on aluminium sheet. The mobile phase developed was Toluene: ethyl acetate: formic acid (3:1.6:0.4), chamber saturation time 20 minutes, migration distance 70 mm, scanning speed about 20 nm/sec and the source of radiation was a deuterium lamp. Sample was prepared by using methanol as a solvent.

The standard solution of Gallic acid was prepared by dissolving 7.5 mg accurately weighed Gallic acid and was dissolved in methanol and volume was make-up to 100 ml with methanol in volumetric flask. The method was validated for linearity, accuracy, limit of detection, limit of quantification, inter and intra-day assay precision, repeatability of measurement and repeatability of sample application.

Development of HPTLC method for Piperine in Triphala-Guggulu Vati and ingredients

The standard piperine was purchased from Sigma-Aldrich USA through Sigma-Aldrich Corporation Bangalore, India. All the chemicals and reagents used were of Analytical grade. The standard solution of piperine was prepared by accurately weighing 1 gm of formulations and separately powdered crude drug of *piper longum* were refluxed with 60 ml of methanol for 1 hour. The extract was filtered and the marc was again refluxed with 40 ml of methanol for another 1 hour. The previous filtrate was filtered and combined. The methanol extract was concentrated under vacuum till a semisolid mass was obtained. It was finally dissolved and the volume made up to 100 ml with methanol and filtered through sintered glass funnel (G-2) by vacuum filtration assembly. The filtrate was centrifuged at 2000 rpm for 30 minutes, the supernant was collected and volume was made up with methanol. The instrumentation and chromatographic conditions were

Spotting device:	Linomat V Semi sample spotter; Camag
Syringe:	100 µL Hamilton
TLC chamber:	Glass twin through chamber (20x10x4 cm); Camag
Densitometer:	TLC scanner 3 linked to win cats software V.4.06; Camag
HPTLC plates:	10 x10 cm, 0.2 mm thickness Pre-coated with silica gel 60
Wavelength:	342.8 nm
Solvent system:	N-hexane: ethyl acetate: methanol (3:4:3)
Temperature:	25^0C

Calibration curve of piperine was prepared by preparing the stock solution of piperine in methanol. Different volumes of stock preparation were spotted on TLC plates. The data of peak areas plotted against corresponding concentration were treated by least-square regression analysis method validation. Repeatability of the sample application and measurement of peak area were carried out using many replicates of the same spot was expressed in terms of percent relative standard deviation (% RSD). The intra and inter day variation for the determination of piperine was carried at three different concentration levels of 200, 400, 600 ng/spot.

Development of HPTLC method for guggulosterone in Triphala-Guggulu Vati and ingredients

The standard E and Z guggulosterone was purchased from Chromadex, CA, USA through LGC PROMOCHEM private limited, Bangalore, India. All the chemicals and reagents used were of Analytical grade. The standard solution of E and Z guggulosterone was prepared by accurately weighing 1 gm of formulations and separately powdered crude drug of *Commiphora wightii* were refluxed with 60 ml of methanol for 1 hour. The extract was filtered and the marc was again refluxed with 40 ml of methanol for another 1 hour. The previous filtrate was filtered and combined. The methanol extract was concentrated under vacuum till a semisolid mass was obtained. It was finally dissolved and the volume made up to 100 ml with methanol and filtered through sintered glass funnel (G-2) by vacuum filtration assembly. The filtrate was centrifuged at 2000 rpm for 30 minutes, the supernant was collected and volume was made up with methanol. The instrumentation and chromatographic conditions were

Spotting device: Linomat V Semi sample spotter; Camag
Syringe: 100 µL Hamilton
TLC chamber: Glass twin through chamber (20x10x4 cm); Camag
Densitometer: TLC scanner 3 linked to win cats software V.4.06; Camag
HPTLC plates: 10 x10 cm, 0.2 mm thickness Precoated with silica gel 60
Wavelength: 254 nm
Solvent system: Toluene: Ethyl acetate: Formic acid: Methanol (6:2:1.5:0.5)
Temperature: 25^0C

Calibration curve of E and Z-guggulosterone was prepared by preparing the stock solution of E and Z-guggulosterone in methanol separately. Different volumes of stock preparation were spotted on TLC plates. The data of peak areas plotted against corresponding concentration were treated by least-square regression analysis method validation. Repeatability of the sample application and measurement of peak area were carried out using many replicates of the same spot was expressed in terms of percent relative standard deviation (%RSD). The intra and inter day variation for the determination of E and Z guggulosterone was carried at three different concentration levels of 200, 400, 600 ng /spot.

Method validation

Validation is concerned with assuring that a measurement process produces valid measurements. Results from method validation can be used to judge the quality, reliability and consistency of analytical results. It is an integral part of any good analytical practice. A measurement process producing valid measurements for an intended application is fit for purpose. Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. Analytical methods need to be validated or revalidated, before their introduction into routine use. Whenever the conditions change for which the method has been validated (e.g., an instrument with different Characteristics or samples with a different matrix); and whenever the method is changed and the change is outside the original scope of the method.

Nowadays, there are several international renowned organizations offering guidelines on Method validation and related topics.

- American Society for Testing and Material (ASTM)
- Codex Committee on Methods of Analysis and Sampling (CCMAS)
- European Committee for Normalization (CEN)
- Cooperation on International Traceability in Analytical Chemistry (CITAC)
- European Cooperation for Accreditation (EA)
- Food and Agricultural Organization (FAO)
- United States Food and Drug Administration (FDA)
- International Conference on Harmonization (ICH) [7].

Development of stability studies

The purpose of stability study was to provide evidence on the quality of drug substance or drug products varies with time under the influence of a variety of environmental factor such as temperature, humidity and light recommendation for storage conditions. Stability study was designed to increase the rate of chemical degradation or physical change of an active drug substance or drug product by using exaggerated storage conditions[8].

All the selected formulation batches of TGVL, TGVM-I and TGVM-II were subjected to stability studies at accelerated conditions at 45^0C at 75% relative humidity for six month. The sample were withdrawn at 1, 3 and 6 months interval of time and evaluated for previously developed parameter colour, odour, taste, moisture content and content of phytoconstituents i.e. Tannic acid, piperine, Z and E- guggulosterone etc,.

RESULTS AND DISCUSSION

UV spectroscopy Fingerprinting of Tannic acid

The absorbance was measured at absorbance maxima 276 nm, against the reagent blank prepared in similar manner without the Tannic acid. The absorbance maxima and Beer's law limit were recorded and data proved the linearity and obeys Beer's law limit was observed, Table no. and Figure No. The linear co-relation between these concentration (X-axis) and absorbance (Y-axis) were graphically presented and the slope (b), intercept (a) and correlation coefficient (r^2) were calculated out for linear equation (Y= bx + a) by regression analysis using the method of the least square, summarized in Table 2 and 3.

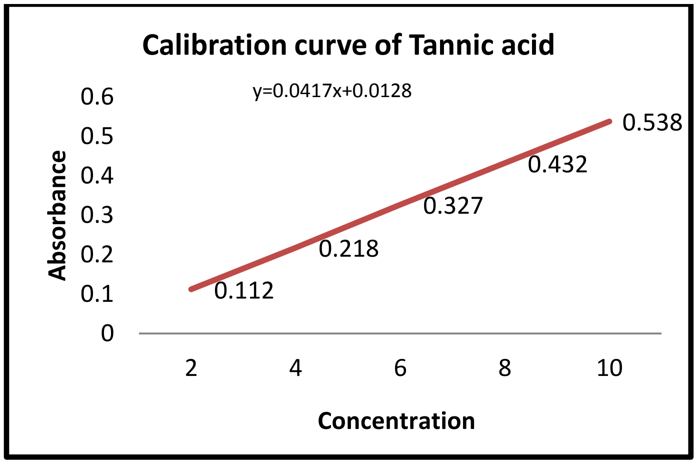

Figure 1: Calibration curve of Tannic acid.

Table 2: Calibration data of Tannic acid.

Concentration in µg/ml	Absorbance
2	0.112
4	0.218
6	0.327
8	0.432
10	0.538

Table 3: **Validation parameter of Tannic acid**.

Parameters	Data of Triphala-Guggulu Vati
Absorption maxima	276 nm
Beer's law limit	2-20µg/ml
Regression equation	$y=0.0417x + 0.0128$
Intercept	0.0128
Slope	0.041682
Co-relation coefficient	$r^2= 0.9995$
Precision	0.251
Accuracy	99.30

Estimation of Tannic acid was done by withdrawing the appropriate aliquots from tannic acid extract of each batch of Triphala-guggulu vati and separately *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis* in 10 ml volumetric flask separately absorbance for aliquots were noted at 276 nm. The corresponding concentration of tannic acid against respective absorbance value was determined using the tannic acid calibration curve. The statistical analysis for checking uniformity in batches is also performed and summarized in Table 4.

The method was validated for precision and accuracy by performing the recovery studies at two level by adding known amount of tannic acid extract of Triphala guggulu vati of which the tannic acid content have been estimated previously. The data obtained and recovery was calculated, which is summarized in Table 5.

Table 4: Estimation of Tannic acid in Triphala-Guggulu Vati.

Name		Tannic acid content % w/w	Confidence level (95%)
Terminalia chebula		14.05±0.29	±0.248
Terminalia belerica		8.7±0.31	±0.232
Emblica officinalis		6.10±0.27	±0.216
Triphala Guggulu Vati	TGVL	9.02±0.39	±0.312
	TGVM-I	8.43±0.42	±0.672
	TGVM-II	7.63±0.68	±0.586

[mean (n=3)±SD]

Table 5: Method Validation and recovery study

Amount of Tannic acid (µg/ml)			RSD %	SE	Recovery %
In Sample	Added	Estimated (n=3)			
100	50	149.23±0.39	0.261	0.159	99.48±0.26
100	100	198.25±0.48	0.242	0.196	99.13±0.24
100	150	246.81±0.24	0.238	0.232	98.85±0.17

Development of UV spectroscopy fingerprinting of Piperine

The absorption maxima and Beer's law limit was recorded and data that prove the linearity and obeys Beer's law were noted. The linear concentration between these concentrations (X-axis) and absorbance (Y-axis) were graphically presented and the slope (b), intercept (a) and co-relation of coefficient (r2) were calculated for the linear equation (Y=bx + a) by regression analysis using the method of least square, summarized in Table 6.

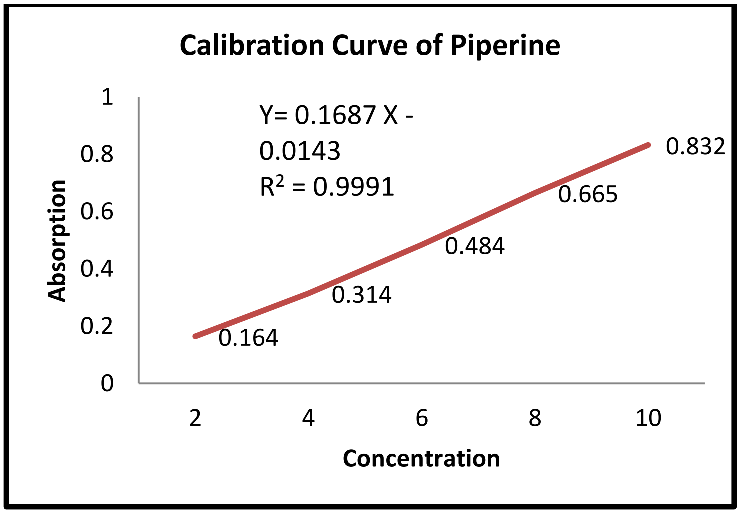

Figure 2: Calibration curve of Piperine.

Table 6: Validation parameter of Piperine.

Parameter	Value
Absorption Maxima	342.8 nm
Beer's law limit	2-10µg/ml
Correlation coefficient	0.9991
LOD	0.410

LOQ	1.40
Regression equation	y=0.1687x-0.0143
Intercept	0.1687
Slope	0.0143
Precision (%RSD)	
Repeatability	0.6275
Intra-day precision	0.83776
Inter-day precision	0.95862

Estimation of piperine was done by preparing appropriate aliquots from piperine extract of all preparations of Triphala-Guggulu Vati and separately of *Piper longum* in 10 ml volumetric flask, separately absorbance for aliquots of each was noted at 342.8 nm. The corresponding concentration of piperine against respective absorbance value was determined using the piperine calibration curve, the estimated piperine content are summarized in Table 7.

The said developed method was validated for precision and accuracy, by performing the recovery studies at two levels by adding known amount of piperine extract of Triphala-Guggulu Vati of which piperine content has been estimated previously. The data was collected and the recovery was determined, which is summarized in Table 8.

Table 7: Estimation of Piperine in Triphala-Guggulu vati and *Piper longum*.

Name		Piperine content % w/w	Confidence level 95%
Piper longum		2.48±0.0057	±0.248
Triphala-Guggulu Vati	TGVL	2.24±0.0024	±0.259
	TGVM-I	1.56±0.0153	±0.251
	TGVM-II	1.68±0.0216	±0.197

Table 8: Recovery Studies of piperine of Triphala-Guggulu Vati.

Amount of Piperine (g/ml)			RSD%	SE	Recovery %
Sample	Added	Estimated			
100	50	149.02±0.61	0.409	0.25	99.34
100	100	197.97±0.292	0.292	0.23	98.98
100	150	247.07±0.195	0.195	0.21	98.82

Development of HPTLC Fingerprinting

Development of HPTLC method for Gallic acid in Triphala-Guggulu Vati and ingredients

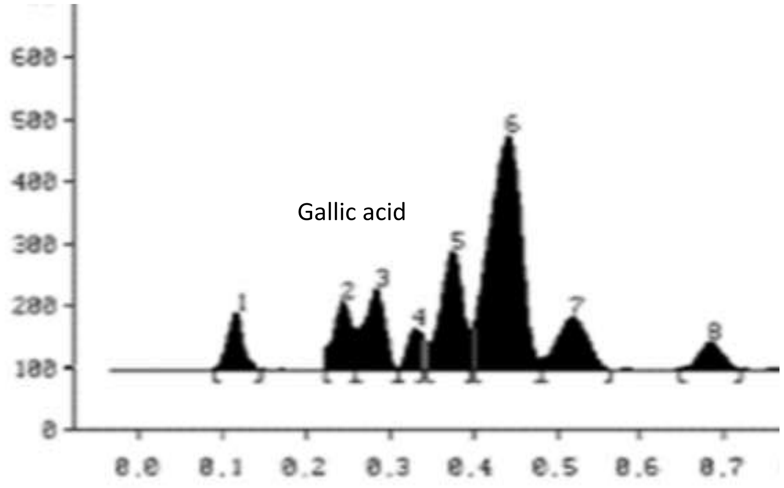

Figure 3: HPTLC chromatogram of Triphala-Guggulu Vati.

and the validation parameters of Gallic acid are summarized in Table 9 and Figure 4. showing the HPTLC chromatogram of standard Gallic acid.

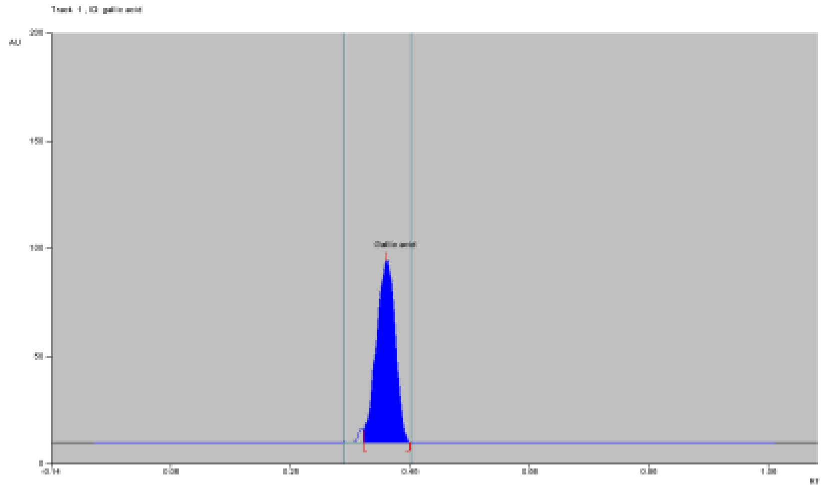

Figure 4: HPTLC chromatogram of Standard Gallic acid.

Table 9: Validation Parameters of Gallic acid.

Parameter		**Value**
Rf		0.38±0.04
Linearity		150-750ng/spot
Correlation coefficient		0.9997
LOD		150
LOQ		0.339
Precision	Inter day	0.64
	Intra day	0.73
Recovery studies	Accuracy	0.35
	Standard error	0.40
	Recovery	99.72
Repeatability of sample measurement		0.642%
Repeatability of sample application		0.479%

Table 10: Estimation of Gallic acid content in Triphala and raw ingredients.

Name		**Gallic acid content % w/w (n=3)**	**Confidence level 95%**
Terminalia chebula		4.63±0.49	±0.47
Terminalia belerica		8.47±0.82	±0.88
Emblica officinalis		3.10±0.41	±0.44
Triphala-Guggulu Vati	TGVL	6.39±0.48	±0.46
	TGVM-I	5.21±0.98	±0.92
	TGVM-II	5.07±1.12	±1.26

The recovery studies were performed at three different levels by adding known amount of Gallic acid to extract of Triphala-guggulu vati of which the gallic acid content have been already estimated. The observations recorded and are summarized in Table 9. The estimation of Gallic acid content was determined by appropriately withdrawing extract of Triphala-guggulu vati and raw ingredients in 10 ml volumetric flask separately. The corresponding concentrations of gallic acid against respective peak area were determined using calibration curve of gallic acid. The results are summarized in Table 10.

Development of HPTLC method for Piperine in Triphala-Guggulu Vati and ingredients

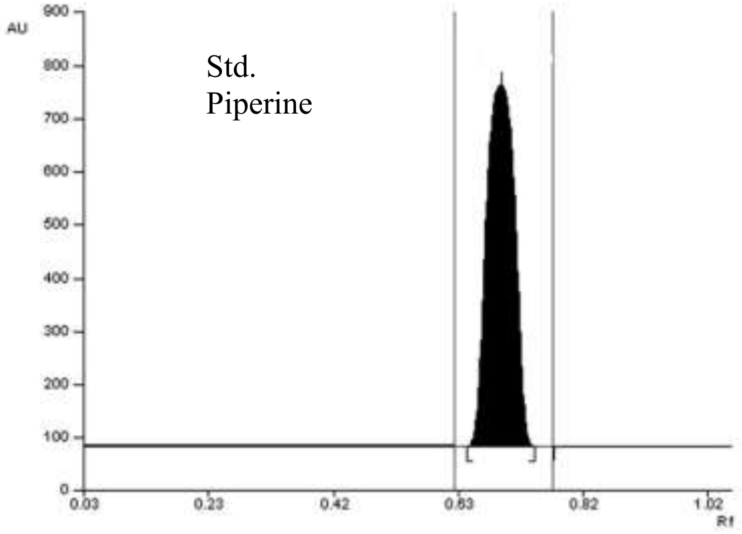

Figure 5: HPTLC chromatogram of standard piperine

Robustness of the method was done at a specific concentration for piperine by introducing small change in the mobile phase volume, duration of mobile phase saturation and activation of pre washed TLC plates with methanol, the effect on the result were examined. In order to estimate the LOD and LOQ blank methanol was spotted LOD was considered 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting the known concentrations of piperine until the average responses were approximately 3 or 10 times the standard deviation of the response determinations. The specificity of the method was confirmed by analyzing the standard drugs and extract. The spot for piperine in the sample was confirmed by comparing the Rf values and spectra with that of both the standard. The peak purity of the piperine was assessed by comparing the spectra at three different levels. For recovery the pre analyzed samples were spiked with extra 50, 100 and 150% of the standard piperine and the mixtures were re-analyzed by the proposed method. The experiment was repeated many a times, Table 11 and Table 12.

Table 11: Recovery for piperine in Triphala-Guggulu Vati

Drug added to analyte	**Concentration found**	**SD** (n=3)	**Recovery**	**% RSD**
50	299.28	±0.5658	99.76	0.1890
100	399.60	±0.5860	99.90	0.1466
150	499.88	±0.0404	99.97	0.0080

Table 12: Validation parameters of method of piperine by HPTLC

Parameters		**Data of Piperine**
Linearity range		200-600 ng/spot
Correlation coefficient		0.9994±0.00034
Limit of detection		2.92
Limit of Quantitation		8.92
Recovery		99.88%
Precision		
	Repeatability of application	0.001986
	Inter-day precision	0.001422
	Intra-day precision	0.023982
Robustness		Robust
Specificity		Specific

For estimation of piperine the appropriate aliquots of piperine extract of each laboratory, marketed batch of Triphala-Guggulu Vati and *piper longum* raw ingredient withdrawn in 10 ml volumetric flask separately. The filtered solution was applied on the TLC plate followed by development and scanning. A single spot at R_f = 0.70 was observed in chromatogram of piperine along with other component. The total piperine content observed in summarized in Table 13.

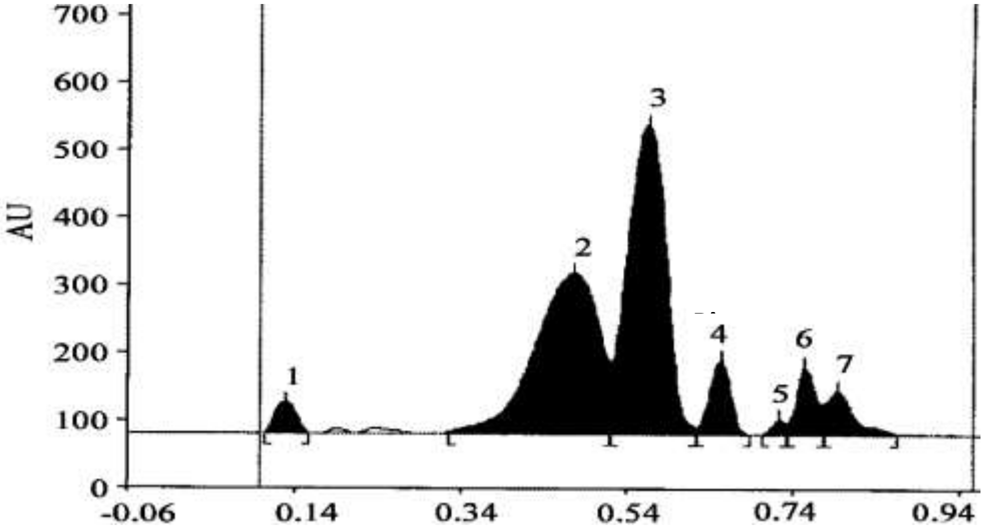

Figure 6: HPTLC chromatogram of Piperine in Triphala-Guggulu Vati.

Development of HPTLC method for Guggulosterone in Triphala Guggulu Vati and its ingredients.

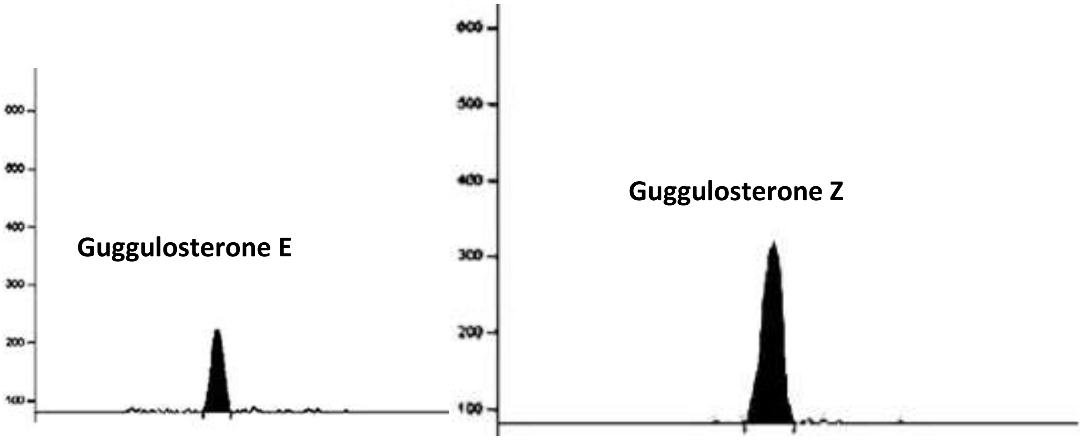

Figure 7: HPTLC chromatogram of standard E and Z guggulosterone

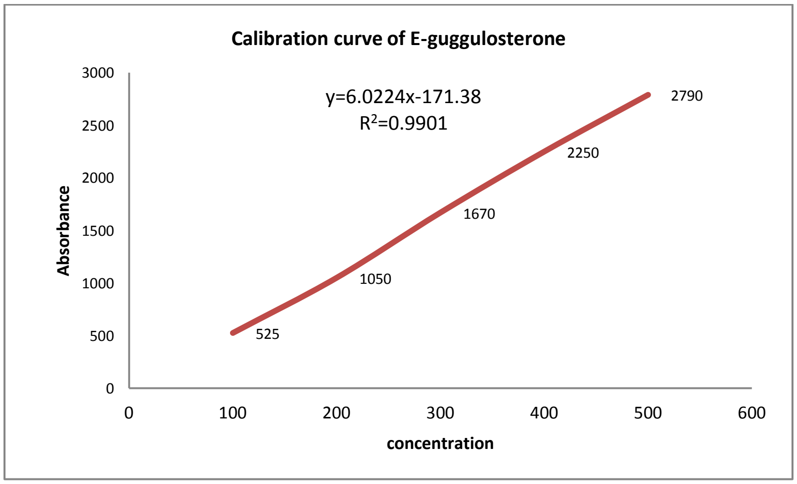

Figure 8: Calibration curve of E – guggulosterone.

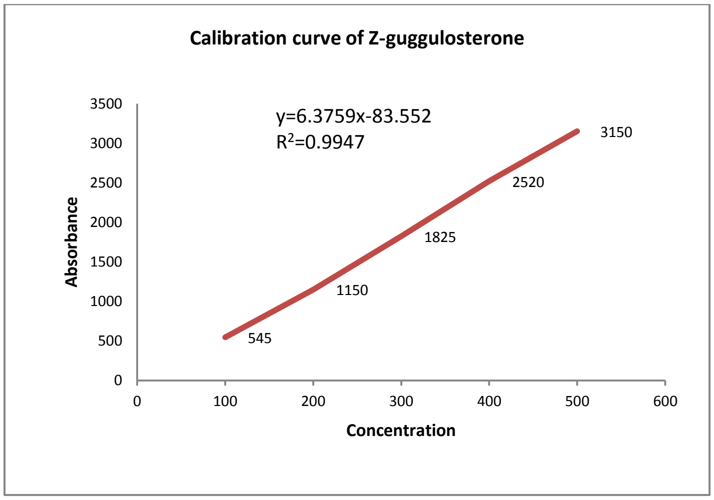

Figure 9: Calibration curve of Z-guggulosterone.

Robustness of the method was done at a specific concentration for E and Z guggulosterone by introducing small change in the mobile phase volume, duration of mobile phase saturation and activation of pre washed TLC plates with methanol, the effect on the result were examined. In order to estimate the LOD and LOQ blank methanol was spotted LOD was considered 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting the known concentrations of E and Z guggulosterone until the average responses were approximately 3 or 10 times the standard deviation of the response determinations. The specificity of the method was confirmed by analyzing the standard drugs and extract. The spot for E and Z guggulosterone in the sample was confirmed by comparing the R_f values and spectra with that of both the standard. The peak purity of the E and Z guggulosterone was assessed by comparing the spectra at three different levels. For recovery the pre analyzed samples were spiked with extra 50, 100 and 150% of the standard E and Z guggulosterone and the mixtures were re-analyzed by the proposed method. The experiment was repeated many a times, Table 14 and Table 15.

Table 13: Piperine content in formulation and crude drug.

Sample	**Piperine content**	**SD** (n=3)	**RSD**
Piper longum	2.18	±0.0256	0.0972
TGVL	1.877	±0.0152	0.0862
TGVM-I	1.446	±0.0173	0.0987
TGVM-II	1.539	±0.0114	0.0828

Table 14: Recovery for E and Z guggulosterone in Triphala-Guggulu Vati

Drug added to analyte	**Concentration found**		**SD**		**Recovery (%)**		**% RSD**	
	E	**Z**	**E**	**Z**	**E**	**Z**	**E**	**Z**
50%	298.95	297.65	±0.548	±0.490	99.65	99.21	0.37	0.82
100%	399.85	398.15	±0.254	±0.550	99.96	99.53	0.39	0.65
150%	498.90	499.05	±0.758	±0.340	99.78	99.81	0.34	0.86

Table 15: Validation parameters of method of E and Z guggulosterone by HPTLC

Parameters	E-guggulosterone	Z-guggulosterone
Linearity range	100-500 ng/spot	100-500 ng/spot
Correlation coefficient	0.9901	0.9947
Limit of detection	20 ng/spot	20 ng/spot
Limit of Quantitation	70 ng/spot	80 ng/spot
Recovery	99.79	99.51
Precision		
Repeatability of application	0.00152	0.00136
Intra-day precision	0.01584	0.01495
Inter-day precision	0.01492	0.01505
Robustness	Robust	Robust
Specificity	Specific	Specific

For estimation of E and Z guggulosterone the appropriate aliquots of Guggulosterone extract of each laboratory, marketed batch of Triphala-guggulu vati and *Commiphora wightii* raw ingredient withdrawn in 10 ml volumetric flask separately. The filtered solution was applied on the TLC plate followed by development and scanning. A single spot at R_f = 0.46 of E and single spot at R_f =0.60 of Z guggulosterone was observed in chromatogram of Guggulosterone along with other component. The total E and Z guggulosterone content determined is summarized in Table 16.

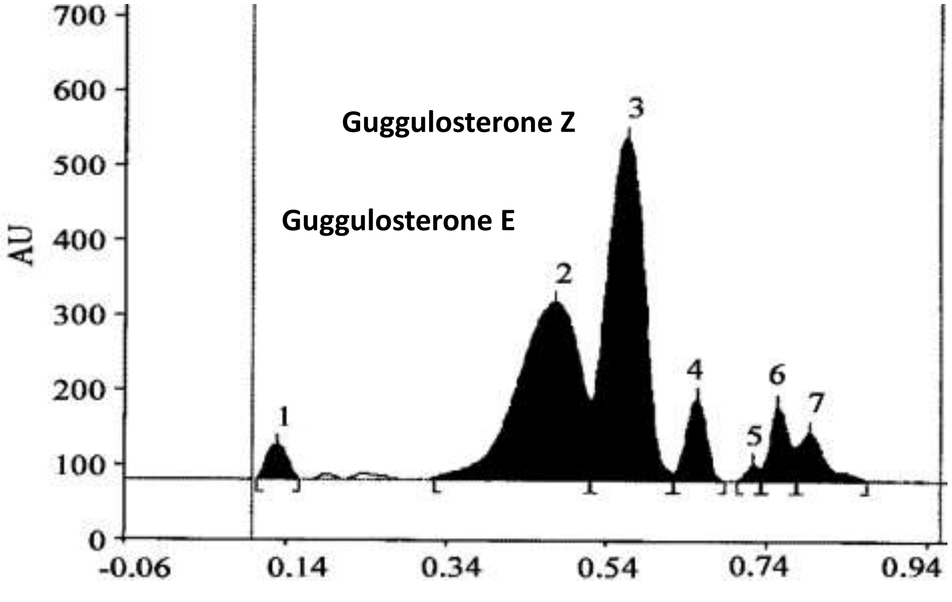

Figure 10: HPTLC chromatogram of E and Z guggulosterone in Triphala-Guggulu Vati.

Table 16: E and Z guggulosterone content in formulation and crude drug.

Sample	Guggulosterone content %		SD (n=3)		RSD	
	E	**Z**	**E**	**Z**	**E**	**Z**
C. wightii	16.21	25.38	±0.015	±0.068	0.0015	0.0051
TGVL	15.34	23.16	±0.029	±0.015	0.0041	0.0028

TGVM-I	13.30	21.10	±0.075	±0.035	0.0063	0.0054
TGVM-II	14.05	19.90	±0.015	±0.054	0.0054	0.0015

Development of method for Stability Testing for Triphala-Guggulu Vati.

The Triphala-guggulu vati sample were withdrawn at intervals of time (1, 3 and 6 months) and evaluated by previously developed method for various parameters like colour, odour, taste, moisture content, Gallic acid, tannic acid, piperine content and E and Z- guggulosterone. The changes in value of above parameters with respect to developed parameters were recorded and summarized in Table 17.

Table 17: Stability studies of Triphala-Guggulu Vati.

Parameter	**Duration**	**Accelerated stability testing of formulation**			**Inference**
		TGVL	**TGVM-I**	**TGVM-II**	
Colour	0	Blackish brown	Blackish brown	Blackish brown	No change
	1	NC	NC	NC	
	3	NC	NC	NC	
	6	NC	NC	NC	
Odour	0	Characteristic	Characteristic	Characteristic	No change
	1	NC	NC	NC	
	3	NC	NC	NC	
	6	NC	NC	NC	
Taste	0	Astringent	Astringent	Astringent	No change
	1	NC	NC	NC	
	3	NC	NC	NC	
	6	NC	NC	NC	
Moisture content	0	7.5±0.40	7.9±0.45	6.9±0.56	Very less change
	1	7.45±0.12	7.5±0.25	6.4±0.21	
	3	7.40±0.35	7.2±0.18	6.1±0.11	
	6	7.38±0.18	6.9±0.26	5.9±0.81	
Tannic acid content	0	9.02±0.39	8.23±0.42	7.63±0.68	Very less change
	1	9.00±0.15	8.20±0.35	7.62±0.18	
	3	8.95±0.24	8.10±0.25	7.50±0.21	
	6	8.94±0.18	8.05±0.17	7.35±0.14	
Gallic acid content	0	6.39±0.48	5.21±0.98	5.07±1.12	Very less change
	1	6.36±0.15	5.19±0.35	5.05±0.28	
	3	6.28±0.24	5.10±0.15	4.95±0.64	
	6	6.20±0.17	5.02±0.64	4.80±1.05	
Piperine content	0	2.24±0.0024	1.56±0.0153	1.68±0.0126	Very less change
	1	2.20±0.051	1.50±0.0041	1.65±0.0157	
	3	2.05±0.014	1.35±0.0024	1.58±0.046	
	6	1.95±0.024	1.30±0.0580	1.49±0.075	
E-Guggulosterone content	0	15.34±0.029	13.30±0.075	14.05±0.015	Very less change
	1	15.30±0.015	13.28±0.015	14.00±0.028	
	3	15.21±0.041	13.19±0.023	13.89±0.041	
	6	15.20±0.037	13.04±0.084	13.80±0.019	

Z-Guggulosterone content	0	23.16±0.015	21.10±0.035	19.90±0.054	Very less change
	1	23.08±0.190	21.05±0.025	19.90±0.071	
	3	22.95±0.024	20.92±0.017	19.75±0.024	
	6	22.90±0.017	20.90±0.051	19.45±0.015	

CONCLUSION

For UV spectroscopy of Tannic acid, the content of Tannic acid was found to be 14.05±0.29, 8.7±0.31, 6.10±0.27, 9.02±0.39, 8.23±0.42 and 7.63±0.68 for *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis*, TGVL, TGVM-I and TGVM-II respectively. The tannic acid content in all the formulations shows close proximities with each other and recovery studies (99.15%) are indicative of reproducibility of method. Hence this developed method is simple, sensitive, rapid, precise, reproducible and accurate and can be used for routine fingerprint for estimation of tannic acid in Triphala-Guggulu vati. For UV spectroscopy of Piperine, the correlation coefficient (r2) value was found to be 0.9991 which indicates good linearity between the concentration and absorbance. The content of piperine was found to be 2.48±0.0057, 2.24±0.0024, 1.56±0.0153 and 1.68±0.0216 for *Piper longum*, TGVL, TGVM-I and TGVM-II respectively. The piperine content shows close proximities with each other mainly in Raw material and Laboratory formulation. The recovery studies shows 99.04 % recovery indicative of reproducibility of the method. Hence the present developed method is simple, sensitive, precise, reproducible and accurate and can be used for routine fingerprint for estimation of piperine in Triphala-guggula vati. During development of HPTLC method for Gallic acid, The Gallic acid content shows close proximities within the formulations. The recovery studies were carried out for the accuracy parameter. The study was carried out at three levels by addition of 50, 100 and 150% of standard drug was added and analyzed by the same method. The mean% recovery was found to be 99.72% indicating reproducibility of the method and %RSD within the limit indicates significant precision of the method. The developed HPTLC method is simple, rapid, precise, reproducible, efficient and accurate for routine estimation of gallic acid in Triphala-guggula vati. Development of HPTLC fingerprint of Piperine, The value indicates close proximities within the formulations. The recovery studies were carried out by addition of 50, 100 and 150 % standard drug to pre analyzed sample and again the sample were analyzed. The recovery was found to be 99.88% which indicates reproducibility of method and the mean %RSD was observed within the limit indicating significant precision of the method. The developed method HPTLC method is simple, precise, reproducible, efficient and accurate for routine estimation of piperine in Triphala-guggula vati. For development of HPTLC fingerprint of Z and E-guggulosterone, the estimated values show close proximities between each other. The recovery studies were carried out for the accuracy parameter. The study was carried out at three levels. To the powdered formulation, standard drug of E-guggulosterone was added at 50,100 and 150% levels and the diluted solutions were analyzed by the developed method. The mean % recovery was found to be 99.79%. The % RSD of three levels was also being calculated, which was found to be within the prescribed limits as listed. This shows significant precision of the method. The developed HPTLC method is simple, accurate, reproducible, Precise and efficient for routine estimation of E-guggulosterone in Triphala-guggula vati. To the pre-analyzed powered formulation, standard drug of Z–guggulosterone was added at 50, 100 and 150 % levels, the diluted solution was analyzed by the developed method. The mean recovery was found to be 99.51 % indicating accuracy of the developed method. The %RSD of three levels were calculated which was found to be in limits as listed. This shows significant precision of method. The developed HPTLC method is simple, precise, accurate, reproducible and efficient for routine analysis of Z- guggulosterone in Triphala-guggula vati. Stability studies reveal that there was insignificant change in the content of Tannic acid, piperine, Z- and E-guggulosterone during the Accelerated conditions within the period of six months.

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