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Original Article

Validated high performance thin layer chromatography method for the quantification of bioactive marker compounds in Draksharishta, an ayurvedic polyherbal formulation

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ABSTRACT

Draksharishta is an ayurvedic polyherbal formulation with Draksha (*Vitis vinifera* L., Vitaceae) as chief ingredient prescribed for digestive impairment, respiratory disorders and weakness. These herbal medicines containing biologically active compounds play a significant role. Therefore it is necessary to carry out the chemical standardization of bioactive marker compounds present in the polyherbal ayurvedic formulation like Draksharishta. The aim of the present work was to develop and validate a HPTLC method for determination of gallic acid, catechin and resveratrol in commercially available marketed and in-house prepared formulations of Draksharishta. This is the first report of quantification of bioactive marker compound resveratrol using HPTLC in Draksharishta. The method employed silica gel precoated thin layer chromatography plates with F_{254} as the stationary phase. The respective mobile phases were used to develop the plates which separated bands according to the marker compound. Camag scanner V was used for densitometric scanning. Further, the method was validated according to International Conference of Harmonization (ICH) guidelines. The R_f values of the three marker compounds were measured. Correlation coefficients were calculated from the standard graph of linearity. Accuracy, precision and recovery were all within the required limits. The developed HPTLC methods for bioactive marker compounds present in in-house and marketed formulations were found to be simple, accurate, precise and robust.

Introduction

In the modern pharmacology and drug development the single chemical entity which is present is responsible for the main therapeutic activity of the drug whereas the preparations of Ayurvedic formulations are based on two principles: use of a single herb or use of more than one herb, which is known as poly herbal formulation. In poly herbal formulations the combining effect of different medicinal herbs help to enhance the potency of the formulation termed as "polypharmacy or polyherbalism" (Kumar et al., 2008; Parasuraman et al., 2014). A marker is a chemical compound which may or may not be therapeutically active while biomarkers are therapeutically active compounds present in medicinal herbs (Bhutani, 2000). These ayurvedic poly herbal formulations is a big task involving quality control consistency that will ensure the therapeutic activity of the finished herbal products as claimed by the standard Ayurvedic books and manufactures. However, most of the conclusions drawn in the ayurvedic texts are based on the ancient knowledge and clinical observations; they lack the modern observations by analytical methods during preparation of a drug (Garg and Bhutani, 2008). Hence there is a need for the development of a reliable protocol for quality assessment of the herbal/poly herbal products by using modern scientific analytical tools.

The fruits of *Vitis vinifera* L. Vitaceae, are commonly known as Draksha (raisins) in the Indian sub-continent and are used in traditional ayurvedic medicine to treat respiratory disorders, digestive disorders and general weakness. Draksharishta is an ayurvedic polyherbal alcoholic formulation included in the Ayurvedic Formulary of India in which dried fruits of *V. vinifera* is the chief ingredient. Draksharishta contains 5–10% of self-generated alcohol with the chemical constituents and properties of *V. vinifera* widely studied and reported (API I, 2000; Tiwari and Patel, 2012). *V. vinifera* contains large amount of phenolic compounds such as resveratrol, catechins, epicatechin, quercetin, gallic acid, procyanidins of which resveratrol is the major constituent (Baydar et al., 2004; Galgut et al., 2011). These compounds have been shown to have various

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pharmacological activities like antiviral, anti-inflammatory, antimicrobial and antioxidant, which show favourable effects on human health such as lowering low density lipoproteins, reduction of heart disease, cancer, digestive and respiratory disorders and improving the immune system (Frankel et al., 1993; Mayer et al., 1997; Teissedre et al., 1996). A HPTLC method has been developed and reported for the quantitative determination of gallic acid and catechin in draksharishta (Tiwari et al., 2013). The hyphenated techniques like High Performance Liquid Chromatography (HPLC), Liquid Chromatography–Mass Spectrometry (LC–MS), Gas Chromatography–Mass Spectrometry (GC–MS), and Capillary Electrophoresis (CE) have been used for the determination of resveratrol (Galgut et al., 2011). This is first report of HPTLC method development and its validation for the presence of resveratrol in Draksharishta.

In recent years, the reporting of various active ingredients (*i.e.* marker profiling) have shown to be a useful method for standardization and quality control of various herbal materials, especially when there is a lack of authentic standards for the identification of all active components present in these complex natural products (Lianga et al., 2004; Chen et al., 2006). For raw materials/herbal products, Thin-Layer Chromatography (TLC) and High-performance Thin-Layer Chromatography (HPTLC) has become an efficient analytical tool for their analysis. HPTLC has been widely used for the identity and quality of the botanicals because of its versatility, reliability, high-throughput and cost effectiveness (Di et al., 2003; Larsen et al., 2004). Furthermore, simultaneous analysis of several components in a poly herbal formulation or herbal extracts becomes possible (Patravale et al., 2001; Abourashed and Mossa, 2004).

According to the ICH guidelines (ICH, 1996, 2005) in our present study the validation parameters developed were accuracy, precision, specificity and robustness for gallic acid, catechin and resveratrol in the three batches of in-house formulations and two marketed formulations of Draksharishta.

Experimental

Standards and chemicals

The analytical grades of organic solvents were procured from Merck specialities Pvt Ltd. (Mumbai). Gallic acid (≥99.5% purity) was purchased from Loba Chemie, catechin (>95% purity) was purchased from Natural Remedies and resveratrol (≥99% purity) was purchased from Sigma–Aldrich.

Plant materials and formulations

The herbs used as ingredients in the preparation Draksharishta were procured from Ayurvedic Pharmacy from the local market (Mumbai). It was deposited and authenticated under the supervision of Dr. A. S. Upadhye at Agharkar Research Institute, Pune. The respective voucher numbers were given for each herb as shown in Table 1. Materials were stored in air tight containers. The two marketed formulation of Draksharishta were purchased from the Ayurvedic Pharmacy (Mumbai).

Preparation of Draksharishta

The three batches of in-house formulations of Draksharishta were prepared by the method as given in Ayurvedic Formulary of India, Part-I. Identification of all the individual plant material was done as per Ayurvedic Pharmacopoeia of India. According to the method given in the standard book, the in-house formulation was prepared at lab scale level. After proper crushing, 48.9 g dried fruits of *V. vinifera* was placed in brass vessel and allowed to soak overnight in 1000 ml of water. This material was boiled until the water was reduced to one fourth 250 ml (decoction) of the original. Heating was stopped at this point and decoction was filtered through muslin cloth in a clean vessel. This was followed by adding 200 g of jaggery and stirred properly until homogeneous solution was obtained following a final filtration. Then, to this filtrate 8 g of *Woodfordia fructicosa* (Dhataki flowers) and 1 g of coarsely powdered prakshepa dravyas including *Cinnamomum zeyleynicum* (stem bark), *Eletteria cardamomum* (seeds), *Cinnamomum tamala* (leaves), *Mesua ferrea* (stamens), *Callicarpa macrophylla* (flowers), *Piper nigrum* (fruits), *Piper longum* (fruits), *Embelia ribes* (fruits) was added, stirred well and filtered again and this filtered fluid was placed for fermentation. The fermented preparation was then filtered with muslin cloth and kept in clean bottles and labelled properly. Samples were prepared from these three in-house batches and the two marketed formulations of draksharishta for HPTLC analysis.

Table 1
Authentication of 10 herbs present in the formulation Draksharishta.

Herbs	Code	Part used	Voucher No.
Vitis vinifera	VV	Fruit	F-202
Cinnamomum zeylanicum	CZ	Stem bark	S/B-140
Callicarpa mycrophylla	CM	Flower	I/F-040
Woodfordia fructicosa	WF	Flower	I/F-041
Piper nigrum	PN	Fruit	F-200
Piper longum	PL	Fruit	F-203
Embelia ribes	ER	Fruit	F-209
Mesua ferrea	MF	Stamens	I/F-042
Cinnamomum tamala	CT	Leaves	L-071
Elettaria cardamomum	EC	Seed	F-201

Preparation of test sample

The three in-house and two marketed formulations of 50 ml each were dried on a water bath until the alcohol was completely removed. Then 50 ml of water was added to the residue left behind. It was then subjected to successive solvent extraction, first with hexane (150 ml) followed by chloroform (150 ml) and ethyl acetate (150 ml). For HPTLC analysis, ethyl acetate fraction of the in-house and two marketed formulations was evaporated to dryness and reconstituted with methanol as given in Ayurvedic Formulary of India, Part-I. The concentrations of three in-house and two marketed formulations obtained were 86.5, 92.5, 90.5, 100.5, 74.5 mg/ml respectively. A sample of 10 mg/ml concentration was prepared for all the in-house batches and marketed formulations. 2.0 μl of each formulation were applied on TLC plates for HPTLC analysis.

Preparation of stock solution and working standard solution of gallic acid, catechin and resveratrol

A common stock solution (1 mg/ml) of gallic acid, catechin and resveratrol was prepared by dissolving 10 mg of each in methanol and making the volume of solution up to 10 ml. The working standard solution of 100 μg/ml was prepared for each by diluting 10 times the stock solution with methanol. The aliquots (2–7 ml of gallic acid), (3–8 ml of catechin), (0.5–1 ml resveratrol) were transferred to 10 ml volumetric flasks and diluted to volume with methanol and applied on TLC plates.

HPTLC instrumentation

TLC plates with a dimension of 20 cm × 10 cm pre coated with 0.20 mm layers of silica gel 60 F_{254} (Merck, Darmstadt, Germany) were used for chromatography. Samples were applied as 8 mm

wide bands and 11.3 mm was the distance kept between the two bands by use of sample applicator Camag Linomat V equipped with a syringe of 100 μl capacity. A constant application rate of 150 nLs^{-1} was used. Camag Scanner V controlled by winCATS Planar Chromatography manager software version 1.4.6 was used as a densitometric scanner. The slit dimensions were 6 × 0.45 mm and the scanning speed 20 mm/s. The radiation source used was a deuterium lamp at a wavelength of 254 nm for gallic acid and 280 nm for catechin and 306 nm for resveratrol.

Chromatographic condition

The mobile phase selected was a mixture of toluene, ethyl acetate and formic acid (6:4:0.8, v/v) for gallic acid, toluene, ethyl acetate and formic acid (5:4:1, v/v) for catechin and chloroform, ethyl acetate and formic acid (5:4:1, v/v) for resveratrol. Plate development was done in a Camag 20 cm × 10 cm glass twin-trough chamber. Before insertion of the plate, the chamber was saturated with mobile phase vapour for 5 min at room temperature (25 ± 2 °C), with the solvent front (development distance) being 7 cm. After the TLC plates were developed and dried by using an air dryer, densitometry scanning was performed at a wavelength of λ = 254 nm for gallic acid, λ = 280 nm for catechin and λ = 306 nm for resveratrol.

Calibration curves of gallic acid, catechin and resveratrol and their analysis in formulations

To determine the linearity, calibration curves were plotted. A 10 μl of each concentration range (20–70 μg/ml) was applied on TLC plates to get final concentration 200–700 ng/spot for gallic acid, (30–80 μg/ml) 300–800 ng/spot for catechin and (5–10 μg/ml) 50–100 ng/spot for resveratrol. The densitometry scanning was performed for each standard and the presence of gallic acid, catechin and resveratrol present in the in-house and marketed formulations were quantified by means of calibration plot.

Method validation

Precision

Six replicates of same concentration of gallic acid (300 ng/spot), catechin (300 ng/spot) and resveratrol (60 ng/spot) were used for the determination of instrumental precision and the repeatability of the method was estimated by carrying out intra-day and inter-day precision at three different concentration levels 200, 400 and 700 ng/spot for gallic acid, 300, 500 and 800 ng/spot for catechin and 50, 70 and 100 ng/spot for resveratrol.

Limits of detection and quantification

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank methanol was spotted six times in a similar way to that of the calibration curve and the signal-to-noise ratio was determined. The calculation was based on the standard deviation (SD) of the response and the slope (S) of the calibration curve. The LOD was considered as 3:1 (SD/S) and LOQ as 10:1 (SD/S).

Accuracy and recovery studies

The accuracy of the method was determined by calculating the recovery of gallic acid, catechin and resveratrol in mixture by standard addition method. To measure the accuracy, known amount of standard solutions of gallic acid, catechin and resveratrol were spiked to 80, 100 and 120% of a pre-quantified sample solution and then their response (peak area) was measured and percentage recovery was calculated. Each response was taken as the average of three determinations.

Robustness

The composition of mobile phase was changed slightly and the effects on the results were examined. Toluene, ethyl acetate and formic acid (6.5:4.5:0.8, v/v) for gallic acid while toluene, ethyl acetate and formic acid (5.5:4.5:1, v/v) for catechin and chloroform, ethyl acetate and formic acid (5.5:4.5:1, v/v) for resveratrol were selected and the chromatograms and run. The amount of mobile phase, temperature and duration of saturation were varied at range of +5%. Time from spotting of all the three standards on TLC plate to the development of the plate and the time from development of plate to scanning was varied as 10, 20 and 30 min. Robustness of the method was checked following the same three different concentration levels as mentioned in precision.

Specificity

The specificity of the method was ascertained by analysing reference standard, test sample, diluent and mobile phase. The spot of the each standard in the sample was confirmed by the R_f values of the separated bands with those of the standards. The peak purity of gallic acid, catechin and resveratrol were measured by comparing the spectra at three different levels *i.e.* peak start, peak apex and peak end of the spot.

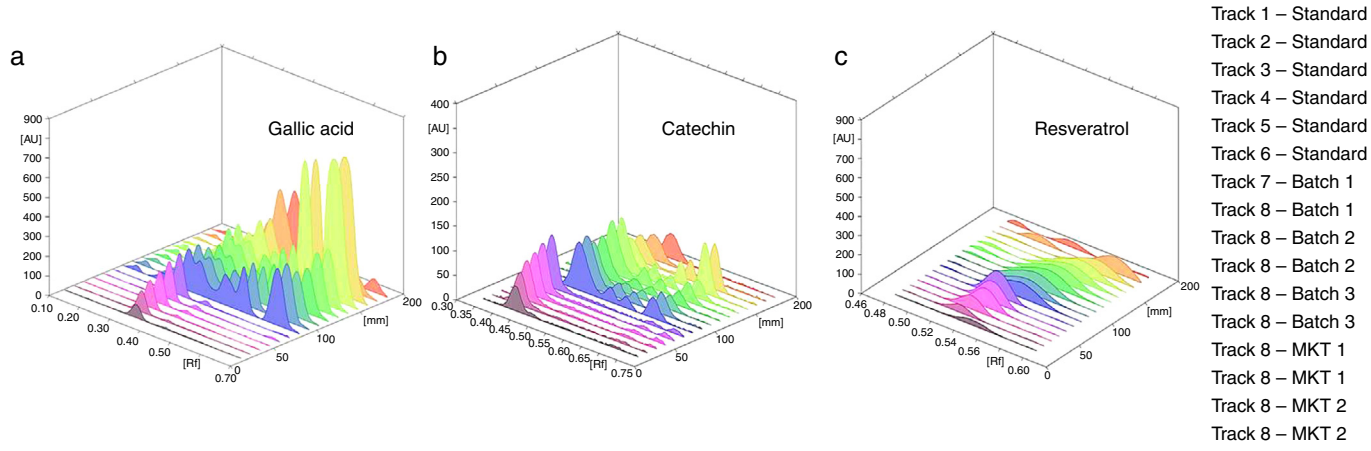
Results and discussion

Optimization of mobile phase

As mobile phase plays a very important role in the chromatographic method, the first step for development of a successful method is to optimize the solvent system for good extraction efficiency. Method that gives dense and compact spots with significant values for determination of gallic acid, catechin and resveratrol in formulations was developed. To optimize the mobile phase, different ratios of Toluene: ethyl acetate: formic acid was studied. Use of toluene, ethyl acetate and formic acid (6:4:0.8, v/v) (Vadivelu and Saraswathy, 2013) resulted in sharp, well defined gallic acid peaks of R_f 0.32 ± 0.02 while solvent system toluene, ethyl acetate and formic acid (5:4:1, v/v) (Dhalwal et al., 2008) resulted in sharp catechin peaks of R_f 0.44 ± 0.02 and chloroform, ethyl acetate, and formic acid (5:4:1, v/v) (Rolfs and Kindl, 1984) resulted in sharp, well defined resveratrol peaks of R_f 0.58 ± 0.02. Before the plate development, the chamber was pre-saturated with the mobile phase for 5 min at room temperature. The three dimensional HPTLC overlay of gallic acid, catechin and resveratrol are shown in Fig. 1.

Calibration curves of gallic acid, catechin and resveratrol and their analysis in formulations

Linearity of an analytical method is its ability, within a given range, to obtain test results that are directly, or through a mathematical transformation, proportional to concentration of analyte (Patel et al., 2011). A good linear relationship between response (peak area) and amount was obtained over the range of 300–700 ng/band for gallic acid at 254 nm, 300–800 ng/band for catechin at 280 nm and 60–100 ng/band for resveratrol at 306 nm. Linear regression data for the calibration plot as correlation coefficients (r) were found to be 0.999, 0.995 and 0.993 respectively tabulated in Table 2 and illustrated in Fig. 2. The data reveals a good linear relationship with the concentration range studied



(a) Chromatogram of gallic acid R_f–0.32 (200–700 ng/spot); (b) Chromatogram of catechin R_f–0.44 (200–700 ng/spot); (c) Chromatogram of resveratrol R_f–.58 (50–100 ng/spot)

Fig. 1. 3D overlay of HPTLC chromatograms of gallic acid, catechin, resveratrol, in-house and marketed formulations.

Table 2
Method validation parameters for the quantification of gallic acid, catechin and resveratrol.

Method property	Gallic acid	Catechin	Resveratrol
R_f	0.32	0.44	0.58
Instrumental precision (RSD [%] $n=6$)	3.0	3.1	3.2
Intra assay precision (RSD [%] $n=6$)	2.7	2.5	3.1
Intermediate precision (RSD [%] $n=6$)	3.5	2.5	3.2
Correlation coefficient, r	0.999	0.995	0.993
Calibration range [ng]	300–700	300–800	60–100
LOD	300	300	60
LOQ	900	900	180
Specificity	Specific	Specific	Specific
Robustness	Robust	Robust	Robust

Table 3
Intra and inter-day precision of HPTLC ($n=6$).

Amount (ng/spot)	Intra-day precision			Inter-day precision		
	Mean area	SD	%RSD	Mean area	SD	%RSD
Gallic acid						
300	2294.2	1.16	0.051	2174.2	0.45	0.021
500	3598.5	1.03	0.028	3526.6	1.53	0.043
700	4267.7	1.32	0.031	4193.3	2.06	0.049
Catechin						
300	1272.9	1.44	0.113	1228.5	1.33	0.108
500	2020.1	1.21	0.059	1983.2	1.12	0.056
800	2849.7	1.09	0.038	2786.4	1.48	0.053
Resveratrol						
60	545.2	0.66	0.121	522.5	0.48	0.092
80	1397.9	1.10	0.078	1296.5	0.77	0.059
100	1960.6	1.25	0.063	1925.7	1.50	0.077

demonstrating its suitability for analysis and also indicated adherence of the method to Beer's law. Gallic acid was found to be 1.767, 1.841, 1.911, 1.361, and 1.595% while catechin was found to be 3.241, 3.142, 3.222, 0.080, and 0.049%, and resveratrol was found to be 0.541, 0.537, 0.538, 0.086, and 0.116% in in-house formulation 1, 2 3, and marketed 1 and 2 formulations, respectively.

Precision

In order to control scanner parameters, that is, repeatability of measurement of peak area, instrumental precision was checked by repeated scanning ($n=6$) of the same spot of gallic acid (300 ng/spot), catechin (500 ng/spot) and resveratrol (80 ng/spot) and were expressed as % RSD and was found to be less than 3% as shown in Table 2, ensuring repeatability of developed method as well as proper functioning of the HPTLC system. The intra-day refers to the use of analytical procedure within a laboratory over a short period of time and inter-day precision involves estimation of variations in analysis when a method is used within a laboratory on different days. The results are shown in Table 3. The method was

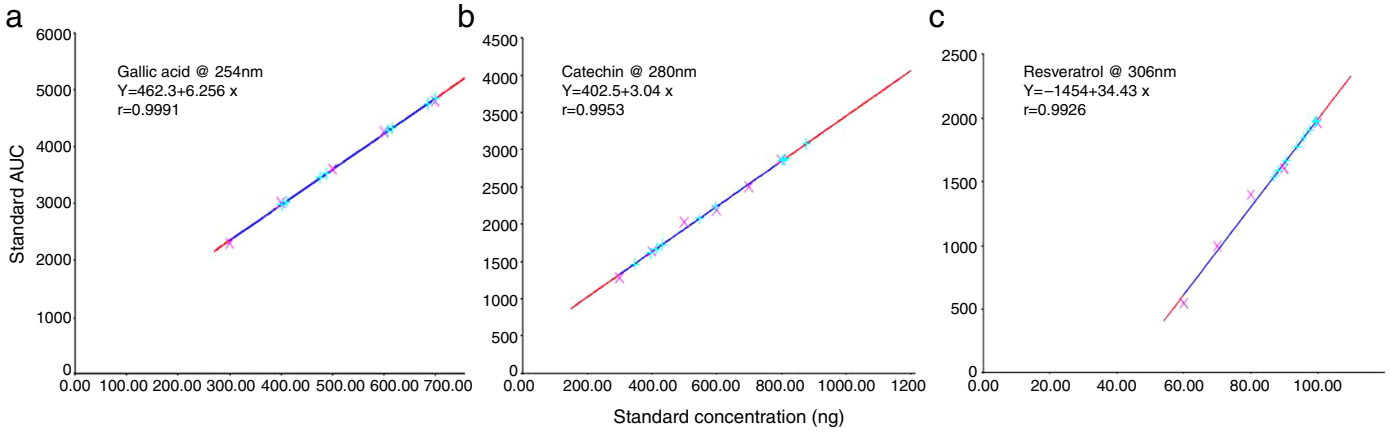


Fig. 2. Calibration plot (a) gallic acid, (b) catechin and (c) resveratrol.

Table 4
Recovery study of gallic acid, catechin and resveratrol.

	Compound	Amount present in sample (μg)	Amount added (μg)	Amount found (μg)	Recovery (%)	Average recovery (%)
	Gallic acid	0.153	0.32	0.421	89.00	
		0.153	0.40	0.489	88.43	87.50
		0.153	0.48	0.538	85.10	
Batch 2	Catechin	0.841	0.40	1.285	103.6	
		0.841	0.50	1.456	108.6	105.70
		0.841	0.60	1.511	104.9	
	Resveratrol	0.047	0.08	0.111	87.26	
		0.047	0.10	0.126	85.80	87.00
		0.047	0.12	0.146	87.90	
	Gallic acid	0.196	0.32	0.471	91.30	
		0.196	0.40	0.535	89.91	89.74
		0.196	0.48	0.594	88.01	
Mkf-1	Catechin	0.338	0.4	0.752	102.02	
		0.338	0.5	0.880	105.05	103.86
		0.338	0.6	0.980	104.52	
	Resveratrol	0.029	0.08	0.087	80.38	
		0.029	0.10	0.111	86.17	85.33
		0.029	0.12	0.133	89.44	

found to be precise based on the results obtained in the intra-day and inter-day precision evaluation study.

Limit of detection and quantification

Detection and quantitation limits with signal-to-noise ratios of 3:1 and 10:1 were considered. Under the experimental conditions employed, limit of detection is the lowest amount of analyte that could be detected was found to be 300 ng/spot for gallic acid and catechin and 60 ng/spot for resveratrol and limit of quantification, the lowest amount of analyte that could be quantified was found 900 ng/spot for gallic acid and catechin and 180 ng/spot for resveratrol as shown in Table 1 which indicates the adequate sensitivity of the method.

Accuracy and recovery studies

Accuracy of an analytical method is the closeness of test results to true value analyte (Patel et al., 2011). It was determined by the application of analytical procedure to recovery studies. The pre-analyzed in-house sample of Draksharishta and its marketed formulation were spiked with 80, 100 and 120% of gallic acid, catechin and resveratrol standard and the mixtures were analyzed again, in triplicate, by the proposed method, to check the recovery of different amounts of these marker compounds. Average recovery for gallic acid, catechin and resveratrol was found to be 87.50, 105.70 and 87%, respectively for in-house sample and 89.74, 103.86 and 85.33%, respectively, for the marketed formulation of Draksharishta as depicted in Table 4. This shows the accuracy of the method in a desired range.

Robustness

The standard deviations of peak areas were calculated for each parameter and %RSD was found to be less than 3%. The low values of %RSD obtained after introducing small deliberate changes in the developed HPTLC method, indicated the robustness of the method. The developed HPTLC method remained to be unaffected by the small but deliberate variations in the experimental parameters, indicating suitability and reliability of the developed method during normal use, thereby indicating the robustness of the method.

Specificity

Specificity is the ability of an analytical method to assess unequivocally the analyte in the presence of sample matrix analyte (Patel et al., 2011). The peak purity was calculated as per regression (r^2). The values for gallic acid was $r^2_{(\text{start, middle})} = 0.9980$ and $r^2_{(\text{middle, end})} = 0.9973$, for catechin $r^2_{(\text{start, middle})} = 0.9969$ and $r^2_{(\text{middle, end})} = 0.9973$ and for resveratrol $r^2_{(\text{start, middle})} = 0.9985$ and $r^2_{(\text{middle, end})} = 0.9990$. Chromatographic specificity was investigated by comparing the R_f value of standards and samples and it was found to be identical. No impurities or degradation products were found along with the peaks of standard drug solutions, hence making the method specific.

Conclusions

The identification and quantification of active ingredients in polyherbal ayurvedic formulations like asavas and arishtas can be evaluated by use of validated analytical methods. A new HPTLC method has been developed for the identification and quantification of gallic acid, catechin and resveratrol in in-house prepared and marketed formulations of draksharishta. Low cost, faster speed, and satisfactory precision and accuracy are the main features of this method. The method was successfully validated as per ICH guidelines and statistical analysis proves that the method is sensitive, specific, repeatable and robust. This method can be conveniently employed for routine quality control analysis of all the three marker compounds for marketed formulations in Ayurvedic/Herbal industry.

Author's contribution

DP (PhD student) contributed in collecting plant sample, formulations, preparation of formulations, performing the laboratory work, *i.e.* chromatographic analysis and drafted the paper. NP designed the study and supervised the overall project work. Authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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