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Research Article

Analytical method development and its validation for simultaneous estimation of catechin and curcumin by HPTLC from ancho lean tablets

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ABSTRACT

HPTLC method which is simple, particular and robust has been developed for the simultaneous estimation of Catechin and Curcumin from an Ayurvedic formulation. The method was validated using parameters such as linearity, specificity, and precision, limit of quantification (LOQ), limit of detection (LOD), accuracy and robustness as per ICH guidelines. The present work deals with development of HPTLC method for simultaneous estimation of catechin and curcumin in marketed Ayurvedic formulations. Chromatographic separation of the drugs was performed on Merck TLC aluminium plates pre-coated with silica gel $60F_{254}$ as the stationary phase. The mobile phase selected was toluene:ethyl acetate: formic acid (7: 2.5: 0.5 v/v/v). The sample solutions were prepared in methanol and linear ascending development was carried out in twin trough glass chamber and scanned at 269 nm using Camag TLC scanner. The two markers were resolved successfully with Rf values 0.23±0.02 and 0.58±0.02 for catechin and curcumin, respectively. The regression analysis data indicated good linear relationship for the calibration plots for Catechin and Curcumin in the range of 1900-2500 ng/spot and 200-800 ng/spot and regression coefficient was 0.990 and 0.997 respectively. The proposed method can be used for the estimation of these markers in combined Ayurvedic formulation.

Introduction

Herbal formulations are moving from side-line to mainstream use with a great number of people pursue remedies and health approaches[1]. Herbal formulations have reached widespread acceptability as therapeutic agents for diabetics, arthritics, liver diseases, cough remedies, memory enhancers and adoptogens[2]. Standardization of polyherbal formulations is obligatorily in order to assess the quality of drugs, depending upon the concentration of the bioactive principles. Quality evaluation of herbal preparation is an integral requirement of industry and other organization dealing with Ayurvedic and herbal products [3].Natural product quality control usually demand a multidisciplinary approach which requires resources and expensive equipment. For identification of crude drugs, it is best to own the authentic reference standard of that specific crude drug. Markers are compound(s) distinctive and characteristic to the plant under investigation and are superiorly present in detectable amounts and can be easily isolated [4]. Phytochemical estimation is one of the tools for the quality assessment, which comprise of preliminary phytochemical screening, chemo profiling and marker compound analysis using advanced analytical techniques. HPTLC has emerged as an essential tool for the qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This encompass developing TLC fingerprint profiles and estimation of chemical markers and biomarkers [5].The following ingredients of Marketed formulation of brand name Sri Sri Ayurveda ANCHO LEAN tablets are as follows: Asana (*Pterocarpus marsupium)* Heartwood, Caldaria (*Bergerakoenigii)* Leaf, Lashoona (*Allium sativum)* Bulb, Trivrut (*Operculinaturpethum)* Root, Haritaki (*Terminalia chebula*) Fruit pulp- 100mg each, Haridra (*Curcuma longa)* Rhizome, Khadirasara (*Acacia catechu)* Extract- 150mg each. The above drugs have been processed in the extracts of Vata (*Ficusbenghalensis)* Bark, Karanja (*Pongamiaglabra)* Bark, Vrikshamla (*Garcinia gummigutta)* Fruit, Varuna (*Crataevanurvala)* Bark- 50mg each.Catechin is a flavonol, known for its antioxidant activity, majorly found in the plant Khadirasara (*Acacia catechu)*

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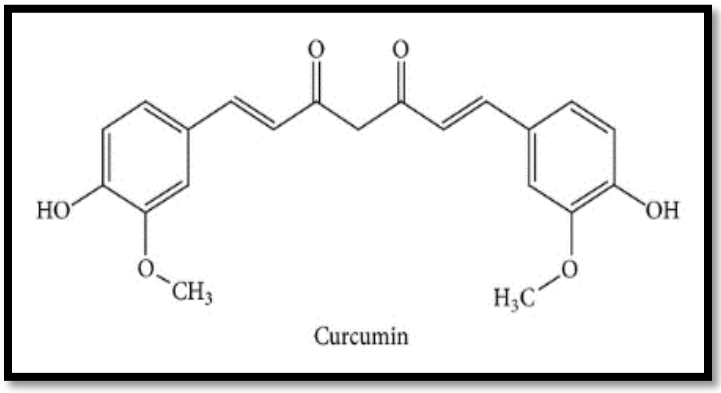
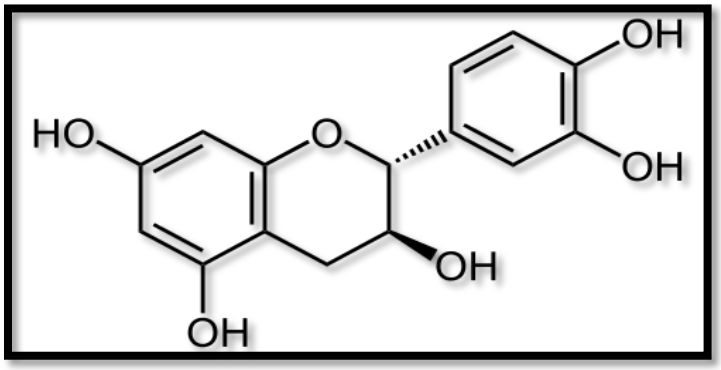
belonging to the family Fabaceae. Whereas, Curcumin, the major polyphenol in turmeric spice, known as Haridra (*Curcuma longa)* belonging to the family Zingiberaceae[6].Curcumin inhibits 3T3-L1 differentiation, caused apoptosis, and inhibited adipokine-induced angiogenesis of human umbilical vein endothelial cells. Addition of curcumin with the high-fat diet of mice did not affect food intake but reduced body weight gain, adiposity and microvessel density in adipose tissue. Curcumin causes rising of 5#AMP-activated protein kinase phosphorylation, reduced glycerol-3-phosphate acyl transferase-1, which led to development of oxidation and decreased fatty acid esterification [7]. Catechin, is the important and major flavonol present in plant, was found to activity which significantly suppress increases in body weight and showing anti-obesity actions by decreasing white adipose tissue weight [8]. Recent research shows that both the compounds inhibits glucosidase and lipase activities, which causes reduction in the intestinal absorption of carbohydrates and lipids and thus shows anti-diabetic and anti-obesity activity [9, 10]. Many techniques have been developed in many literature studies for the determination of Catechin and Curcumin individually or in combination with other markers. Whereas, there is no HPTLC method reported for the simultaneous estimation of these markers in combined formulation. The aim of this developed and validated method is to expand HPTLC method with UV detector for the simultaneous estimation of Catechin and Curcumin in an ayurvedic proprietary formulation.



(a)



(b)

Fig. 1: Structures of (a) Curcumin and (b) Catechin

Materials and Method

Marketed formulation

Marketed formulation of brand Sri Sri Ayurveda name ANCHO LEAN tablets was procured from local market of Mumbai, Maharashtra, India.

Standards and Reagents

All the chemicals of LR grade were procured from S.D. fine chemicals, Mumbai, Maharashtra, India. Analytical standards of Curcumin and Catechin were purchased from Yucca Enterprises, Mumbai.

Instrumentation

Chromatographic separation was attained on HPTLC plates using Camag (Muttenz,Switzerland) Linomat V sample applicator equipped with 100µl Hamilton syringe. TLC scanner 3 with win CATS software was used for detection of samples.

HPTLC Method Development

Preparation of standard solution

Stock solutions of Catechin and Curcumin (1000µg/ml) were prepared separately by dissolving 10 mg of accurately weighed standard in 10 ml of methanol.

Preparation of working solution

Working solutions were prepared from the stock solutions. Solution of each markers having concentration 100µg/ml were prepared from the 1000 µg/ml stock solution. From these further dilutions were made to get solutions of 95-125 µg/ml (1900-2500 ng/spot) and 10-40 µg/ml (200-800 ng/spot) for Catechin and Curcumin respectively.

Preparation of sample solution

2gm of powder weighed approximately by triturating tablets and extracted with 30 ml of methanol using rotamantle for 30min by maceration method. The solution was further cooled and filtered to get methanolic extract. 1 ml of the above extract was diluted to 10 ml with methanol and used for further analysis.

Chromatographic conditions

Chromatographic separation was achieved on precoated HPTLC plates with silica gel 60 F_{254}.Standard solutions of both the markers and samples (extract of the formulation) were applied to the plates as bands of 6.0 mm wide, 10.0 mm from the bottom edge of the chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat 5 sample applicator equipped with a 100μl Hamilton syringe. Ascending development to a distance of 80mm was performed at room temperature (25 ± 2°C), with mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried and then scanned at 269 nm with a Camag TLC Scanner 3 using the deuterium lamp with winCATS software.

Calibration curves for Catechin

Serial dilutions were made in the concentration range of 95-125µg/ml and 10-40µg/ml for catechin and curcumin, respectively. Aliquot of above solutions (20µl) were applied with the band width of 6 mm, in triplicate on TLC plate (10×10 cm) to obtain a concentration range of 1900-2500ng/spot for catechin and 200-800ng/spot for curcumin. Peak area for each band was recorded. Separate calibration curves were obtained by plotting a graph of peak area vs. concentration of catechin and curcumin.

HPTLC Method Validation

The method was developed and validated as per ICH guidelines Q2 (R1) for parameters which are as follows: linearity, specificity, precision, Limit of detection, Limit of quantitation, accuracy and robustness.

Linearity

Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. It was obtained by plotting peak area Vs concentration of standard and finding regression coefficient(r^2).

Specificity

Specificity is the ability to assess the analyte in the presence of components that may be expected to be present in the sample matrix. The specificity of the method was ascertained by comparing the Rf value and the peak purity was assessed by comparing the spectrum of standard catechin and curcumin with sample.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. As per the ICH guidelines precision should be performed at three different levels: Lower Quality Control (LQC), Medium Quality Control (MQC) and Higher Quality Control (HQC). Intra-day precision exhibit the precision under the same operating conditions over a short interval of time. It is assessed by using at least 9 determinations over the specified range for the procedure. The intra-day precision was performed 3 times on same day, while inter-day precision was performed on 3 different days.

Limit of detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Limit of Quantitation (LOQ)

The quantitation limit of analytical procedure is the smallest amount of analyte in a sample which can be quantitatively evaluated with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

The LOD and LOQ are expressed as:

$LOD = 3.3\ \sigma/S$

$LOQ = 10\ \sigma/S$

Where, σ = Standard deviation of response, S = Slope of the calibration curve both of them are obtained from the calibration curve of the individual maker compound.

Accuracy (Recovery)

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. As per ICH, Accuracy should be assessed using a minimum of 9 determinations over a minimum of three concentration levels covering the specified range i.e. 3 concentrations levels in triplicate. The accuracy of the method was examined by performing recovery experiments by the standard addition method. The recovery of the drugs at different levels in the formulations was checked by spotting the test samples of known concentration of catechin and curcumin simultaneously on the plates. The spots were then spiked in three different concentrations (80%, 100% and 120% w/w) by further adding known amount of standard mixture of catechin and curcumin. These samples were then

analysed and the results obtained were compared with expected results.

Robustness

The robustness of an individual analytical procedure is a measure of its capacity to remain unaffected by small, but intentional changes in method parameters and provides an evidence of its reliability during normal usage. The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. Following the introduction of small changes in the mobile phase composition (±0.2 ml for major component), the effect on the results was examined. The amount of mobile phase was varied over the range of ± 5%. The saturation time of development chamber was varied by ± 5 min. The robustness of the method was determined at two concentration levels (95 and 110 µg/ml) for catechin and (10 and 25 µg/ml) for curcumin.

Results and Discussion

Selection of Analytical wavelength

In situ HPTLC spectral overlain of catechin and curcumin were taken. Isoabsorptive point was found at 269 nm and was selected as the scanning wavelength.

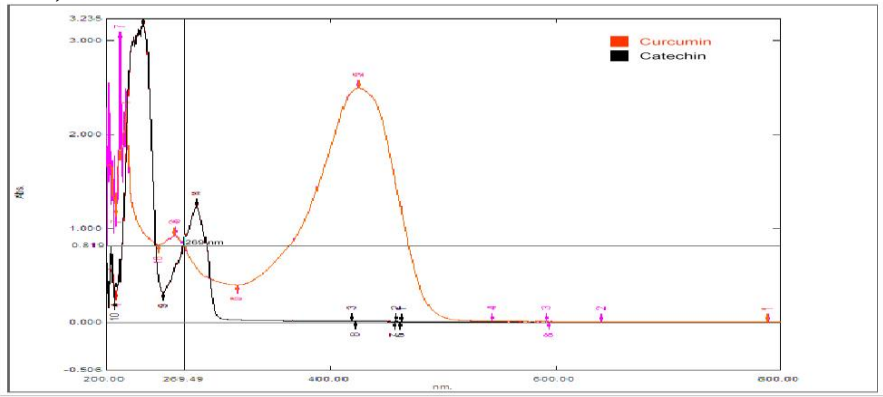


Fig. 2: HPTLC overlain spectra of Catechin and Curcumin

Optimization of Chromatographic conditions

The HPTLC experimental conditions such as mobile phase composition and wavelength of detection were optimized to provide precise, reproducible and accurate results for the determination of catechin and curcumin. The mixed standard stock solution containing 100µg/ml of catechin and curcumin was spotted on the TLC plate and developed in different solvent systems. Good resolution and sharp peaks were obtained with minimum tailing by using mobile phase consisting of Toluene: Ethyl acetate: Formic acid in the ratio 7: 2.5: 0.5 (v/v/v/v). Catechin and Curcumin were satisfactorily resolved with Rf values at 0.23±0.02 and 0.58 ±0.02, respectively.

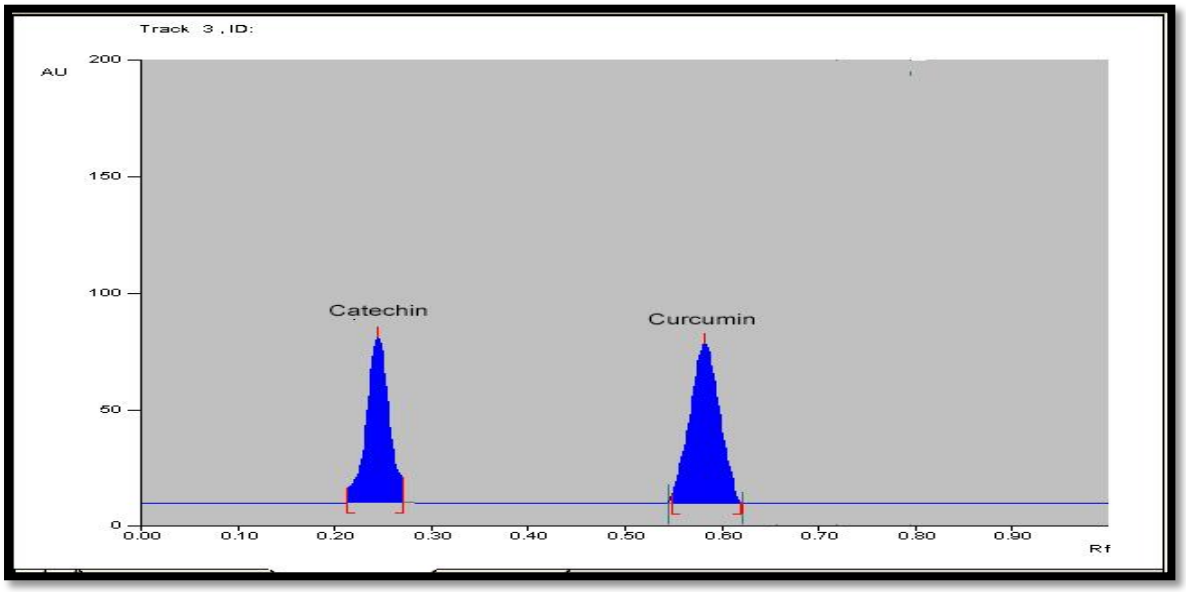
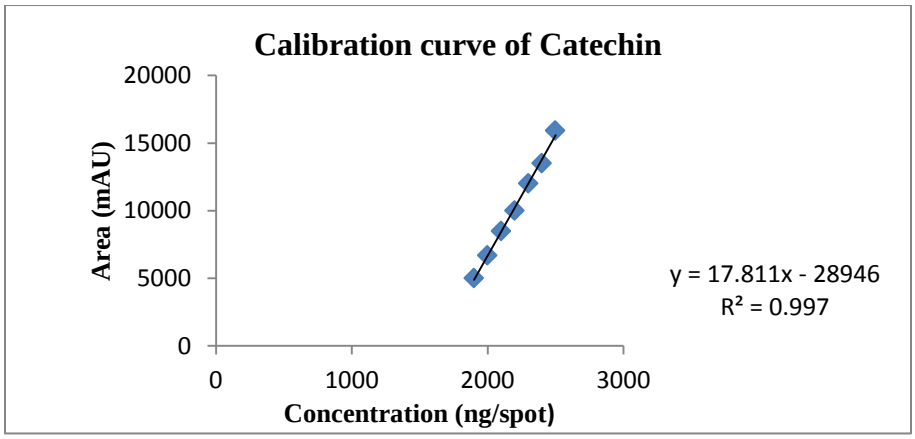


Fig. 3: Chromatogram of standard Catechin [Rf: 0.25±0.02] and Curcumin [Rf:0.58±0.02]

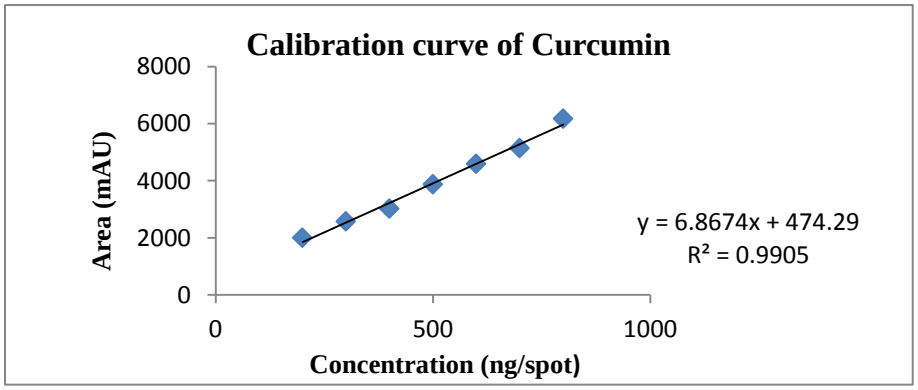
HPTLC Method Validation[11]

Linearity

Linear relationship was observed by plotting drug concentration against peak area for each compound. Catechin and Curcumin showed linear response in the concentration range of 1900-2500 ng/spot and 200-800 ng/spot, respectively. The linearity was validated by the high value of the correlation coefficients. The results are tabulated in Table 1.



(a)



(b)

Fig. 4: Calibration curve of Catechin (a) and Curcumin (b)

Table 1: Linear regression data for calibration plot for Curcumin and Catechin

Parameters	Catechin	Curcumin
Linearity range (ng)	1900-2500 ng/spot	200-800 ng/spot
Regression equation	y = 17.81x-28946	y = 6.867x+474.2
Correlation coefficient (r^2±S.D.)	0.997	0.990
Slope (mean ± S.D.)	17.81	6.867
Intercept (mean± S.D.)	28946	474.2

S.D. = Standard deviation.

Specificity

It was observed that other constituents present in the extract did not interfere with the peak of catechin and curcumin. Therefore the method is specific. The spectrum of standard catechin and curcumin corresponds with sample.

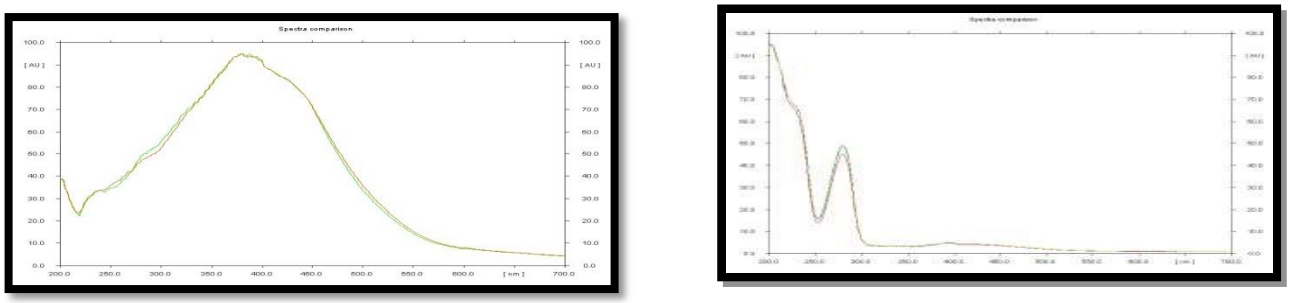


Fig. 5: (a) Overlay spectra of standard catechin and catechin from tablet extracts. (b) Overlay spectra of standard curcumin and curcumin from tablet extracts.

Precision

Intraday precision is used to describe the variation of the method, at three different concentration levels within the same day while interday precision is for variation between different days. The % RSD values for both intraday and interday precision were found within acceptable limits as shown in Table 2 respectively.

Table 2: Intra-day and Inter-day precision results

	Intra-day precision			Inter-day precision	
Marker	**Concentration (ng/spot)**	**Standard Deviation**	**% RSD**	**Standard Deviation**	**% RSD**
Catechin	1900	93.46	1.78	97.39	1.88
	2200	206.47	1.95	86.67	0.81
	2500	212.62	1.38	96.22	0.63
Curcumin	200	24.45	1.25	29.13	1.53
	500	55.06	1.43	34.83	0.92
	800	75.75	1.22	43.87	0.70

Accuracy (Recovery)

The recovery of catechin from marketed formulations, Ancho lean tablets was found to be 96.38% while that of curcumin from the same formulation was found to be 101.88% as shown in the table 3 and table 4.

Table 3: Results of accuracy studies for Catechin

Formulation	**Level of recovery (%)**	**Theoretical content of marker (ng/spot)**	**Amount of marker recovered (ng/spot)**	**% Recovery**	**Average % recovery**
Ancho lean tablets	80	15296.95	15166.21	99.14	
	100	16997.1	16058.44	94.47	96.38
	120	18696.81	17866.40	95.55	

Table 4: Results of accuracy studies for Curcumin

Formulation	**Level of recovery %**	**Theoretical content of maker (ng/spot)**	**Amount of marker recovered (ng/spot)**	**% Recovery**	**Average % recovery**
Ancho lean tablets	80	1438.36	1448.33	100.69	
	100	1598.18	1635.14	102.31	101.88
	120	1757.99	1804.65	102.65	

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ were found to be 18.04ng/spot and 54.68 ng/spot for Catechin and 13.980 ng/spot and 42.42ng/spot for Curcumin, respectively.

Robustness

The % R.S.D of the peak area was calculated for changes in mobile phase composition and duration of saturation time in triplicates of both the markers individually. This method was found to be robust as the statistical data shows that % RSD of the peak areas obtained was less than 2 %.The concentrations 1900 and 2200ng/spot for catechin whereas 200 and 500ng/spot for curcumin were used to study the robustness parameter. The values of % R.S.D were less than 2% as shown in (Table 5) which demonstrates that the developed method is robust.

Table 5: Robustness results for Catechin and Curcumin

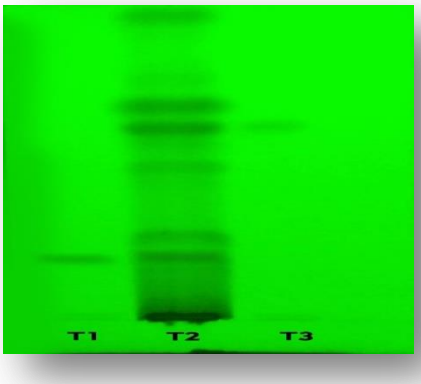
Parameter	Deviation	% RSD			
		Catechin		Curcumin	
		Area		Area	
		1900 ng/spot	2200 ng/spot	200 ng/spot	500 ng/spot
Mobile phase composition	Toluene: Ethyl acetate: formic acid 7.5: 2.0 : 0.5	1.00	0.73	1.73	1.95
	Toluene: Ethyl acetate: formic acid 6.5: 3: 0.5	1.01	1.47	0.63	1.35
Saturation time	15 mins	0.96	1.13	1.55	1.72
	25 mins	0.82	0.87	1.1	1.38

Analysis of marketed formulations

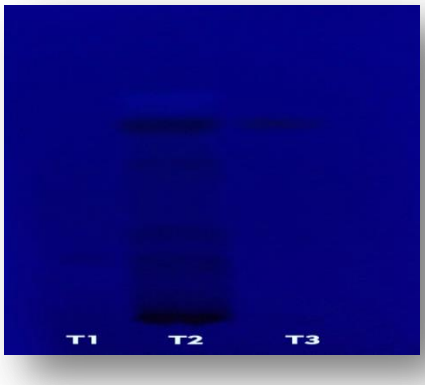
The developed method was applied for detection and quantification of catechin and curcumin in marketed formulations namely Ancho lean tablet. The peaks for catechin and curcumin were observed at Rf values 0.25±0.02 and 0.60±0.02, respectively in the densitogram of the extracts. There was no interference from other compounds present in the extracts. The total content of catechin and curcumin present in the marketed formulations is as shown in table 6.

Table 6: Catechin and Curcumin content in polyherbal formulations

Formulation	% w/w content	
	Catechin	Curcumin
Ancho lean tablets	0.637	0.059



(6.a) At 254 nm



(6.b) At 366 nm

Figures 6.a and 6.b HPTLC profiles of standard catechin, curcumin and marketed formulation extract at 269nm

T1: Standard catechin. T2: Extract of Formulation. T3: Standard curcumin

Conclusion

The developed and validated method was found to be accurate, simple, precise and reliable for the detection and quantification of catechin and curcumin and was successfully established for evaluation of Ancho Lean tablets. This can be used as a standard technique for routine, rapid and accurate quantitative determination of catechin and curcumin in the drug as well as marketed formulations. Catechin and Curcumin showed good resolved spots with selected and optimized mobile phase. This can be used as a standard technique for routine quantitative determination of Catechin and Curcumin in the marketed formulations. This validated HPTLC method can be used to evaluate both the markers from any marketed formulation. Thus this method conforms to the requirement of ensuring quality and safety of an Ayurvedic medicines.

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