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Research Article

DEVELOPMENT AND VALIDATION OF A NOVEL HPTLC METHOD FOR SIMULTANEOUS ESTIMATION OF EMBELIN AND CATECHIN

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ABSTRACT

A new simple, precise, rapid and selective high-performance thin-layer chromatographic (HPTLC) method have been developed for the simultaneous determination of embelin and catechin in herbal formulations containing cashew leaves and amla fruit powder. Two herbal formulations were prepared in-house as anti-oxidant and anti-bacterial medicine, one of which is tablet and second is cream. These formulations were analyzed by developed and validated HPTLC method for two main phytoconstituents (Catechin and Embelin). This method shows well resolved peak of embelin and catechin. The results obtained in validation assays indicate that the method is linear, accurate, précised and robust. This developed and validated method is well applied for the analysis of the in-house formulations, which in-turn can be applied in large scale. This routine analysis will take our herbal medicine to the global level.

KEY WORDS: Catechin, Embelin, HPTLC, *Anacardium officinalis, Embilca ribes,* tablet, cream

INTRODUCTION

Standardization of herbal medicines is a topic of study as well as a tool to improvise and upgrade traditional use of plants as medicine. The plant based medicines are complex in terms of their content and the chemical identity of each constituent. This is one of the reasons why the standardization is not carried out as routine quality control testing by the manufacturer of herbal medicines. Secondly, even the regulatory bodies in India do not demand for the same. Thus, despite of all the benefits with minimal side effects plant based medicines do not run with the part of allopathic products in the market.

It is standardization which will bring the effective, well known promising herbal medicines in the race of global pharmaceutical market. This can be achieved by the analytical methods which are developed and validated by following certain well stated guidelines such as International conference on harmonization (ICH). Evaluation of traditionally used medicinal plants and products by the proven and well accepted (by the regulatory bodies) methods of quality control is in demand. Thus standardization has become an essential step for the acceptance of herbal medicines at global level and marker based standardization is one of the most sophisticated method.[1-5]

Emblica officinalis (syn:Phyllanthus emblica) Amla, family Euphorbiaceae or Indian gooseberry, is a well known fruit used as home remedy as well as many manufacturers as has been used as anti-oxidant, blood sugar controller.[6]

Anacardium occidentale, commonly known as Cashew, and is a member of the family Anacardiaceae. Catechin is present as one of the vital bioactive constituents in Cashew and has very well antioxidant property .[7]

As a combination of these two active antioxidant herbal plants, we had formulated tablet as antioxidant supplement and antibacterial cream. The bioactive markers in these formulations are mainly embelin (from amla powder) and catechin (from cashew leaves powder). Thus, we have attempted to standardize these formulations by HPTLC using embelin and catechin as biomarkers. This standardization will help to upgrade our in house product and will able to study more in detail the stability of these formulations.

MATERIAL AND METHOD

Solvents and chemicals

Biomarkers catechin and embelin were purchased from Yucca Enterprises Maharashtra India. The in-house formulations (tablets and cream) were analysed. All chemicals and reagents used were of analytical grade and purchased from Rankem and S. D. Fine Chemicals, India.

HPTLC Instrumentation

The sample solutions were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica gel aluminium plate $60F_{254}$ (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat V (Switzerland). The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography. A constant application rate of 1.0 µL/s was employed and space between two bands was 5 mm. The slit dimension was kept at 5mm × 0.45 mm and 10 mm/s scanning speed was employed. The slit bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of toluene: ethyl acetate: formic acid (4.5.5:0.5) (v/v) and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ^{0}C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8 cm. Subsequent to the scanning, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed with Camag TLC scanner III in the reflectance-absorbance mode at 254 nm and operated by Win CATS software (1.3.0 Camag). Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression.

Standard Solutions

Standard stock solutions (1 mg/mL) of reference catechin and embelin were prepared in methanol. Working solutions of catechin and embelin were prepared by appropriate dilutions of the stock solutions with methanol. All solutions were prepared freshly prior to analysis.

Preparation of Sample Solutions

Twenty tablets containing cashew leaves extract and amla fruit extract were powdered and 200 mg of powdered tablet was dissolved in 100 mL methanol. The solution was filtered and the filtrate was used for analysis.

1 gm of cream was suspended in 100 mL methanol. The suspension was sonicated for 30 min and then centrifuged for 15 min at 3000rpm.

A constant application volume of 10.0 µL/spot was employed for all the sample solutions.

Assay Validation

The proposed HPTLC method was validated according to the International Conference on Harmonization guidelines.[8-16] All measurements were performed in triplicates.

Calibration studies

Linearity was evaluated in the range of 500-2500 ng / spot and 1000-3000 ng / spot for catechin and embelin respectively. Peak area *versus* concentration was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration curve were determined. LOD and LOQ were determined by using standard deviation method. Detection limit $=3.3\sigma /S$ and quantitation limit$=10\ \sigma /S$ where σ is the residual standard deviation of a regression line and S is the slope of the calibration curve.

Precision studies

Precision of the method was evaluated by repeatability (intra-day) and instrumental precision. Each level of precision was investigated by three sequential replicates of injections of catechin and embelin at concentrations of 150 ng/spot, 300 ng/spot and 450, 900 ng/spot respectively.

Accuracy studies

In order to evaluate the validity of the proposed method, accuracy of the method was determined the percentage recoveries of known amounts of mixture of catechin and embelin added to sample containing a mixture of all three standards. The analyzed samples were spiked with 80, 100 and 120 % of median concentrations (1500ng/spot catechin and 2000 ng/spot embelin) of standard solution in a solution containing the 100 % of respective concentration of two standards. Accuracy was calculated from the following equation:

[Practical concentration/ theoretical concentration] × 100.

Robustness

For the determination of the robustness of method, chromatographic parameters, such as mobile phase composition and detection wavelength, were intentionally varied to determine their influence on the retention factor and quantitative analysis.

The mobile phase composition was altered by ± 5 % changes in the composition of methanol. The two composition of ethyl acetate were tried 5.775 (+5% of 5.5) and 5.225 (-5% of 1.5) were tried. The chamber saturation time was altered from 15 min to 30 min.

Table 1: LOD and LOQ of both markers

Markers	LOD	LOQ
Catechin	300	900
Embelin	150	450

LOD : ng/spot

Table 2.1: Results of Precision Studies of Catechin

Type of Precision	Intra-day			Inter-day		
	AUC for concentration of Catechin			AUC for concentration of Catechin		
Sr. No	**1500**	**2000**	**2500**	**1500**	**2000**	**2500**
Mean	834.2	1107.54	1405.12	750.1	1204.10	1511.02
% RSD	0.19	0.26	0.74	0.64	0.58	0.32

AUC concentration of Catechin : ng/spot

Table 2.2: Results of Precision Studies of Embelin

Type of Precision	Intra-day			Inter-day		
	AUC for concentration of Embelin			AUC for concentration of Embelin		
Sr. No	**1000**	**1500**	**2000**	**1000**	**1500**	**2000**
Mean	1395.42	1980.15	2599.01	1379.89	1983.80	2564.17
% RSD	0.89	0.76	0.44	0.78	0.42	0.70

AUC concentration of Embelin : ng/spot

Table 3.1: Recovery studies of Catechin

In 2000 ng Catechin	**Mean AUC of catechin**			**Recovery ± S.D. (%)**
	In sample	**In standard**	**In spiked samples**	
1600 (80%)	939.09	986.25	2025.04	105.17 ± 0.91
2000 (100%)	1117.01	1120.52	2156.71	96.38 ± 0.75
2400 (120%)	1302.1	1314.01	2616.01	95.60 ± 0.66

Table 3.2: Recovery studies of Embelin

In 2000 ng Embelin	**AUC of embelin**			**Recovery ± S.D. (%)**
	In sample	**In standard**	**In spiked samples**	
1200 (80%)	1579.08	1602.11	3204.67	100.74 ± 1.11
1500 (100%)	1900.25	1955.14	3855.39	94.72 ± 0.95
1800 (120%)	2399.01	2487.20	4626.77	96.24 ± 1.07

Table 4.1: Robustness (Mobile phase variation) studies of Catechin

Sr. No	Mobile phase composition			R_f	AUC
	Toluene	**Ethyl acetate**	**Formic acid**		
1.	4.2	5.5	0.5	0.59	1142.05 ± 0.11
2.	3.8	5.5	0.5	0.59	1142.15 ± 0.27
3.	4	5.775	0.5	0.59	1140.07 ± 0.74
4.	4	5.225	0.5	0.59	1142.01 ±0.38
S.D	-	-	-	0.0	1.00

Mobile phase composition : v/v

Table 4.2: Robustness (Chamber saturation time) studies of Catechin

Sr. No	Chamber saturation time	R_f	AUC
1.	15	0.59	1145.11 ± 0.11
2.	20	0.59	1142.99 ± 0.27
3.	25	0.59	1143.77 ± 0.74
4.	30	0.59	1141.52 ±0.38
S.D.	-	0.0	1.5

Chamber saturation time: min

Table 4.3: Robustness (Mobile phase variation) studies of Embelin

Sr.No.	**Mobile phase composition**			**R_f**	**AUC**
	Toluene	**Methanol**	**Formic acid**		
1.	4.2	5.5	0.5	0.39	1987.11 ± 0.28
2.	3.8	5.5	0.5	0.39	1984.99 ± 0.58
3.	4	1.625	0.5	0.39	1989.58 ± 0.42
4.	4	1.375	0.5	0.39	1986.88 ± 0.63
S.D	-	-	-	0.0	1.88

Mobile phase composition : v/v

Table 4.4: Robustness (Chamber saturation time) studies of Embelin

Sr. No.	Chamber saturation time	R_f	AUC
1.	**15**	0.12	1985.71 ± .36
2.	**20**	0.12	1988.32 ± .52
3.	**25**	0.12	1985.08 ±0.47
4.	**30**	0.12	1987.11 ± 0.22
S.D.	-	0.0	1.45

Chamber saturation time : min

Table 5: Stability Studies of solution containing two markers

Marker compounds	**Temperature**					
	4 °C			**25 °C**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Catechin	98.97	98.57	98.19	98.17	97.77	97.41
Embelin	97.68	98.91	98.48	99.04	98.74	98.23

Table 6: Percent Content of Catechin and Embelin in in-house formulations containing cashew leaves and Amla fruit powder

Extract & Formulation	**Percent Content ± S.D. (%)**	
	Catechin	**Embelin**
Tablet	00.574±0.24	00.314 ±0.19
Cream	00.412 ±0.11	00.298 ±0.21

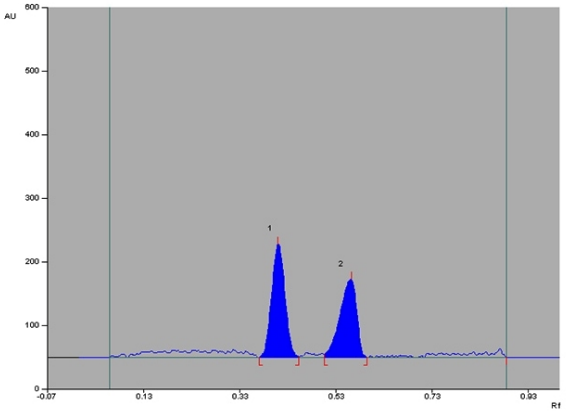

Figure 1: HPTLC profile of standard Embelin and Catechin

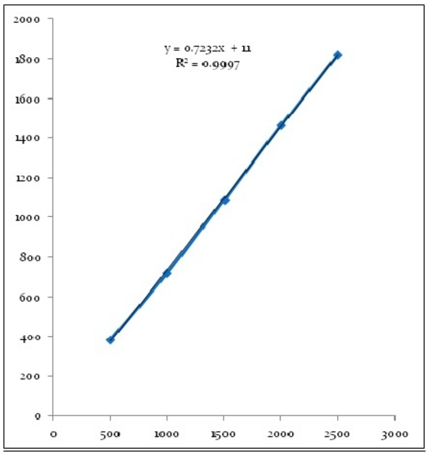

Figure 2: Linearity graph of Standard Catechin

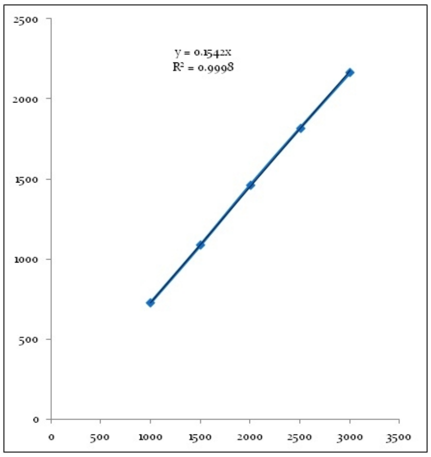

Figure 3: Linearity graph of standard Embelin

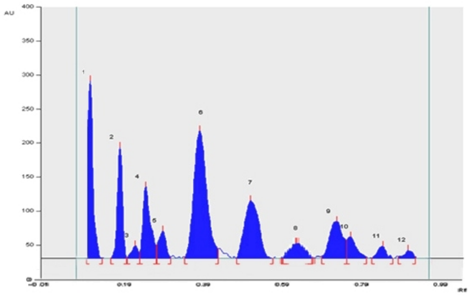

Figure 4: HPTLC profile of in-house tablet formulation

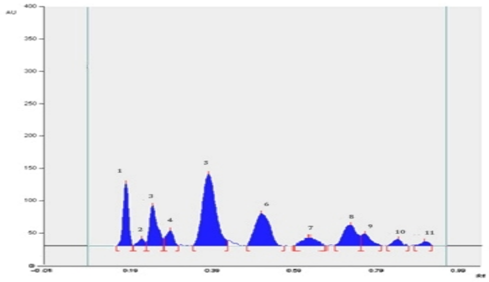

Figure 5: HPTLC profile of in-house cream formulation

RESULTS AND DISCUSSION

Method Optimization

The proposed method gave very good separation and resolution of all the two markers as indicated in **Table 1 and Figure 1**.

Method Validation

Linearity, limit of detection and quantitation

Under the above described experimental conditions, linear correlation between the peak area and applied concentration was found to occur in the concentration range of 84-504 ng ml^{-1} for kaempferol, 168-1008 ng ml^{-1} for rutin, and 800 -1800 ng ml^{-1} for quercetin. The correlation coefficient of kaempferol, rutin and quercetin was found to be 0.9997, 0.9998 and 0.9998 respectively. The peak area (y) is proportional to the concentration of respective marker and the regression equations as following:

For Catechin, **$y = 0.7232x + 11$ (Figure 2)**

For Embelin, **$y = 0.1542x$ (Figure 3)**

The experimentally derived LOD and LOQ for all three markers were determined as mentioned in **Table 1.**

Precision

Precision data on repeatability (intra-day) and instrumental variation for three different concentration levels are summarized in **Table 2.1 and Table 2.2**. Precision studies showed R.S.D. less than 1%, indicating a good precision

Accuracy

The sample containing 1500ng/spot catechin and 2000 ng/spot embelin were spiked with the known amount of standard, and the percent ratios between the recovered and expected concentrations were calculated. Recoveries were obtained in the range of **95.86-99.24%** and 97.25-98.12 %, depicting the HPTLC proposed method for simultaneous estimation is accurate for the quantification of two marker compounds. (**Table 3.1 and Table 3.2)**

Robustness

No changes were observed in retention time and peak shape of both the markers with the changes made with mobile phase and chamber saturation time (**Table 4.1**, **Table 4.2, Table 4.3**, **Table 4.4 and Table 5**). The resolution and the separation of markers were also unaltered

Analysis of in-housed formulations containing cashew leaves and amla fruit

Validated method was applied to standardization for both the in-house formulations. The shape of the peaks was not altered by other substances present in the matrix. The developed and validated HPTLC method gave well resolved peaks of two respective markers in both the formulations **(Figure 4 and 5).** The percent content of catechin and embelin in two products each of two plants are indicated in **Table 6.** The content of each marker is also calculated for per unit of each dosage form.

CONCLUSION

The rapid, simple, precise, accurate and reproducible HPTLC method was successfully developed and validated for simultaneous analysis of both marker compounds in two different plant products. The other ingredients of each product did not affect the analysis. Thus, this method can be applied for a wide range for plant based medicines ranging from traditional to modern dosage form. Thus application of this method commercially can uplift the global acceptance of traditional systems medicine of India.

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