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RESEARCH ARTICLE

HPTLC Method Development and Validation for Simultaneous Estimation of Berberine, Ellagic Acid and Ferulic Acid in *Amrtadi Churna*

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Abstract

The objective of the current study was to develop a new simple and precise High Performance Thin Layer Chromatography (HPTLC) method for standardization of three biomarkers i.e., berberine, ellagic acid and ferulic acid in *Amrtadi churna*. *Amrtadi churna* is mainly used for hyperacidity and contains dried plant parts of *Gokshur*, *Amla* and *Guduchi*. The method was developed and validated using precoated silica gel at 60 F_{254} as the stationary phase and toluene:ethyl acetate:formic acid:methanol (6:6:1.6:0.4, v/v/v/v) as the mobile phase. The detection and quantification were performed at 319 nm and the R_f value obtained was 0.35±2 for berberine, 0.5±2 for ellagic acid and 0.74±2 for ferulic acid. The method was validated as per ICH guidelines in terms of linearity, precision, specificity, accuracy and robustness.

Keywords: *Amla, Gokshur, Guduchi*, ICH, Standardization

1. Introduction

Ayurvedic formulations provide basic healthcare treatment for patients with several ailments[1]. As the use of ayurvedic formulations increases, so do increases concerns about their safety and efficacy. Hence, it is essential to provide more information to properly understand the risks related to the use of these products[2]. The World Health Organization also emphasizes quality control of ayurvedic formulations by using modern techniques and providing suitable parameters and standards. The quality of herbal plants and their medicines is mainly defined by the content of effective biomarkers present in them[3,4].

For the proper definition of this term and properly evaluating the dynamic nature of various biomarkers, many chromatographic and spectroscopic techniques were developed. Quality control of ayurvedic formulations is an efficient procedure for pharmaceuticals to ensure the purity and uniformity of marketed pharmaceutical products. So, different tools and techniques are implied to ensure quality[5].

The HPTLC method is useful in a variety of pharmaceutical analysis, including qualitative and quantitative compound and impurity determination, assays, semi-quantitative limit tests, and degradation studies[6]. High-Performance Thin Layer Chromatography (HPTLC) is a chromatographic technique that helps in the proper separation, detection and quantification of active constituents present in plant extracts and their related formulations[7]. HPTLC has a better ability to separate multi-components and is really easy with various coloured chemical constituents. Many sensitive colour reagents can also be used in HPTLC for the detection of spots[8].

Amrtadi churna is a polyherbal formulation that aids in the treatment of hyperacidity (a type of *pitta dosha*) and many other geriatric diseases. *Amrtadi churna* consists of one part each of '*Guduchi*', '*Amla*' and '*Gokshur*'[9].

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Guduchi is the Sanskrit name of *Tinospora cordifolia,* which belongs to the Menispermaceae family. It is a deciduous climbing shrub that often blooms in characteristic greenish-yellow hues. The dried stem of *Tinospora cordifolia* contains berberine, a yellow-coloured bioactive compound. Berberine is an isoquinoline alkaloid with antimicrobial, anticancer, antidiabetic, antiarrhythmic, antiseptic, antibacterial, and anti-inflammatory properties[10-13].

Emblica officinalis or Phyllanthus emblica (Euphorbiaceae) is known as *Amla* or Indian gooseberry. The dried pericarp of *amla* contains ellagic acid as one of the active constituents. Ellagic acid is a phenolic compound possessing antiviral, antimutagenic and antioxidant properties, which helps in treating many diseases[14-16].

Gokshur/*Gokhru* is made from dried *Tribulus terrestris* (Zygophyllaceae) fruits. The fruits contain ferulic acid, one of the constituents having antioxidant, antidiabetic and anticancer properties. Ferulic acid is used in various cosmetics, to protect skin from sunlight and inflammation[17-19].

A literature survey revealed that berberine, ellagic acid and ferulic acid were estimated individually in plant extracts as well as in marketed formulations. But they were not simultaneously estimated in any formulation. Therefore, the study aimed to develop a rapid, precise and reproducible HPTLC method for determining berberine, ellagic acid and ferulic acid in *Amrtadi churna*.

2. Materials and Methods

2.1 Materials and Reagents

The crude drug powders (*Guduchi*, *Amla* and *Gokshur*) and standards (Berberine, Ellagic acid and Ferulic acid) were procured from Yucca Enterprises, Mumbai, India. Silica gel $60F_{254}$ TLC plates (E Merck, Germany) were used as a stationary phase. All chemicals and reagents were of analytical grade and obtained from Suvidhinath Laboratories, Vadodara, Gujarat. Prepared *Amrtadi Churna* was used for analysis.

2.2 Instrument and Apparatus

- HPTLC CAMAG TLC Scanner 4; Software-VisionCATS.
- LINOMAT V sample applicator assisted by continuous pressure of nitrogen gas (4 to 6 kg/cm^2).
- CAMAG Twin Trough Chamber; Dimensions - 10×10 cm; 20×10 cm.
- Pre-coated Silica gel 60 F_{254} TLC Aluminium sheets (20×20 cm); Merck, Germany.
- Electronic digital balance; Reptech, India.
- Ultraviolet (UV) cabinet; UV- 1800; Shimadzu, Japan.
- Ultrasonicator; Inco, Dubai.
- Linomat, Camag (syringe of 100 µL volume).

2.3 Preparation of *Amrtadi churna*

The *Amrtadi churna* was prepared as per the method given in the Ayurvedic Formulary of India (Part III). Identification of all procured material was done according to the Ayurvedic Pharmacopoeia of India. Procured materials were thoroughly cleaned, powdered and mixed properly by passing through sieve 80#. This prepared *churna* was further used for analysis.

2.4 Preparation of Reference Standard Solution

Amounts of 10 mg each of berberine, ellagic acid and ferulic acid were weighed separately and transferred into three different 10 mL volumetric flasks. About 5 mL of methanol was added and dissolved by intermittent sonication and mixing and then the volume was made up to 10 mL with methanol to obtain a final concentration of 1 mg/mL of each component.

2.5 Preparation of Sample Solution

About 1 gm of *Amrtadi churna* was macerated with 10 mL methanol for 24 hours. It was then filtered and the filtrate was completely dried on a water bath. From the dried residue 1mg of sample was then dissolved in 1 mL methanol to obtain a final concentration of 1 mg/mL.

2.6 Method Validation[7]

Validation of the developed method was performed as mentioned in International Council for Harmonisation (ICH) guidelines Q2(R1)[21]. Specificity, precision, linearity, robustness, the limit of detection, and the limit of quantitation were all performed to validate the proposed method in a suitable and efficient way.

2.7 Linearity

A mixture of 1000 µg/mL of all three standards i.e., berberine, ellagic acid and ferulic acid was prepared in methanol. Calibration was performed by determining the range with respect to the concentration of markers present in the formulation. This stock solution was spotted on TLC plates in the volume of 1 µL-5µL. Using the above result, a calibration graph was plotted using concentration versus peak area to obtain a proper regression coefficient.

2.8 Precision

The interday and intraday precision of the developed method was determined by properly analysing standard solution (1 mg/mL) 6 times by setting the same volume for the spot i.e., 3.0 µg/spot. Intraday precision was determined on the same day and interday precision of the method was confirmed by performing the same procedure on different days under the same set of experimental situations. These multiple series of measurements agreed on their closeness and hence helped in calculating the standard deviation between them.

2.9 Robustness

The robustness of the developed method was analysed by making small changes in the saturation time (±2) of the mobile phase and in the detection wavelength (±2) of the spots to calculate the standard deviation and %RSD, thus proving the reliability of the method.

2.10 Specificity

Specificity was resolved by analysing standard and test samples under the developed conditions. By assessing the spectra of the standard compounds at the peak, apex, peak start and peak end positions of the spot, the peak purity was determined.

2.11 Accuracy

The accuracy study of the developed method was done by standard addition. The sample solution was spiked with the biomarkers and then analysed to calculate its average percent recovery and percent relative standard deviation (%RSD). This spiking was done at three concentration levels (80%, 100% and 120%) and was conducted in triplicate.

2.12 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

By properly analysing standard solution (1 mg/mL) by setting different volumes of the spot i.e., 1.0 – 5.0 µL/spot of all three biomarkers, LOD and LOQ were calculated. To analyse the lowest amounts of berberine, ellagic acid and ferulic acid to be determined and quantified.

3. Results and Discussion

3.1 Optimization of HPTLC Conditions

Sample spots of berberine, ellagic acid and ferulic acid were applied to TLC plates and various solvents were used as the mobile phase to separate the three standards. After various trials and evaluating different ratios of solvent, toluene, ethyl acetate, formic acid and methanol was finalized which gave proper separation of berberine, ellagic acid and ferulic acid in both standard and sample as shown in Figure 1. CAMAG TLC scanner 4 was used with the help of Vision CATS software and proper wavelength was selected by analysing the overlay spectrum of all three biomarkers.

Proper separation of peaks and maximum quantification of all three standards together was done at 319 nm wavelength as shown in Figure 2. Many other parameters, like chamber saturation time, plate drying time and UV scanner lamp, were optimised in order to detect the standards properly and effectively. The optimized mobile phase chamber saturation time for the given mobile phase was found to be 20 minutes with 10 minutes of drying time and a deuterium lamp for scanning, as shown in Table 1.

Table 1. Optimized conditions

Parameters	Optimized conditions
Stationary phase	Pre-coated Silica gel 60 F254 TLC Aluminum sheets (20×20;10×20) cm
Mobile phase	Toluene: Ethyl acetate: Formic acid: methanol (6:6:1.6:0.4, v/v/v/v)
Saturation time	20 minutes
Wavelength	319 nm
Lamp	Deuterium

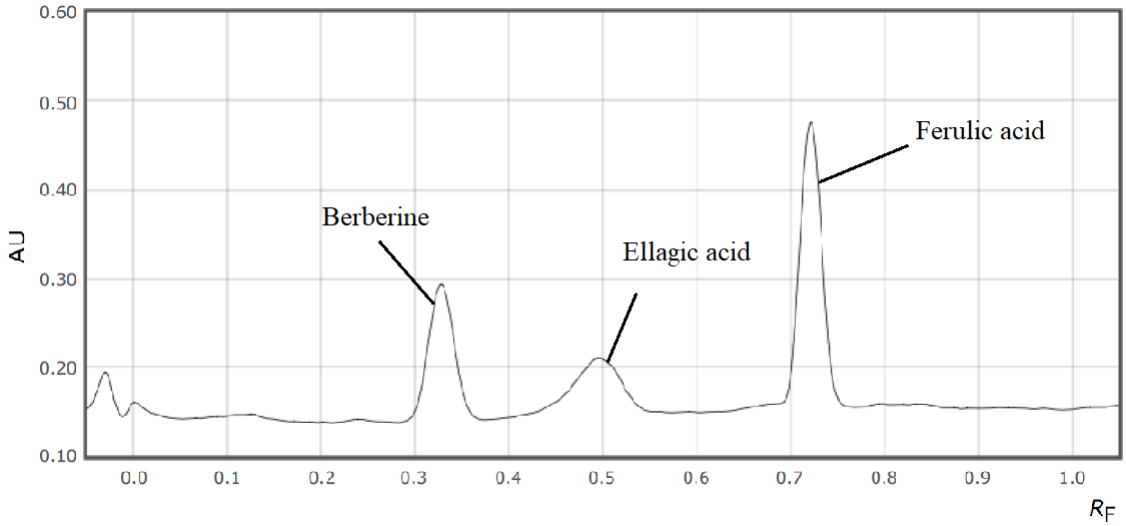

Figure 1. HPTLC chromatogram of Berberine, Ellagic acid and Ferulic acid.

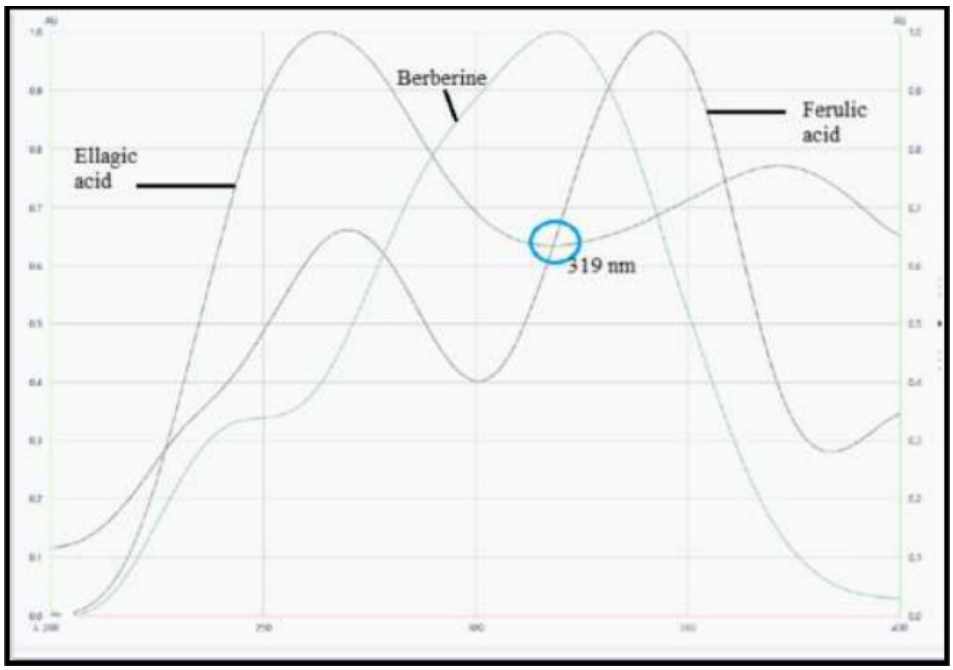

Figure 2. Wavelength selection.

3.2 Validation Parameters

Validation of the developed method using various optimized conditions was performed in accordance with the International Council for Harmonisation (ICH) guidelines[21].

3.3 Linearity

The linearity of the proposed method was estimated by analysing a series of different concentrations, and the calibration curve of concentration versus area was plotted. Table 2 shows parameters like the linearity range, regression equation, R^2 value, intercept, etc. Calibration curves were plotted for the mixture of all three standards, i.e., berberine (Figure 3), ellagic acid (Figure 4) and ferulic acid (Figure 5). Also, the 3D chromatogram of berberine, ellagic acid and ferulic acid reflects linearity over the measured wavelength (Figure 6) and an R^2 value was obtained.

Table 2. Method validation parameters

Parameters	Berberine	Ellagic acid	Ferulic acid
R_f value	0.35±2	0.5±2	0.74±2
Linearity range (ng/ml)	1000-5000	1000-5000	1000-5000
Regression equation	Y=0.000002x+0.0061	Y=0.000003x+0.0044	Y=0.000003x+0.0115
R^2	0.9903	0.9946	0.9883
Intercept	0.0061	0.0044	0.0115
Saturation time (min)	20	20	20
Specificity	specific	specific	specific
Robustness	robust	robust	robust
LOD (ng/mL)	313	178	266
LOQ (ng/mL)	948	540	806
Scanning wavelength (nm)	319	319	319

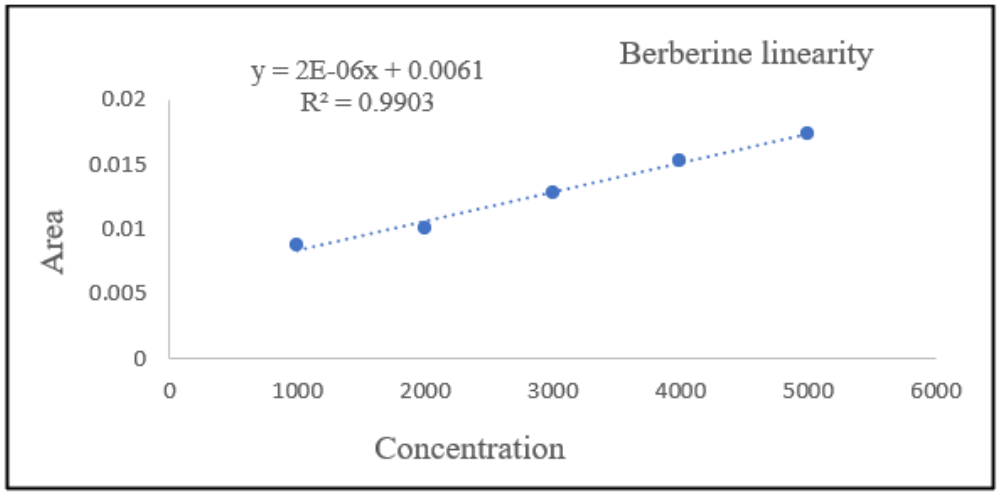

Figure 3. Calibration curve of Berberine.

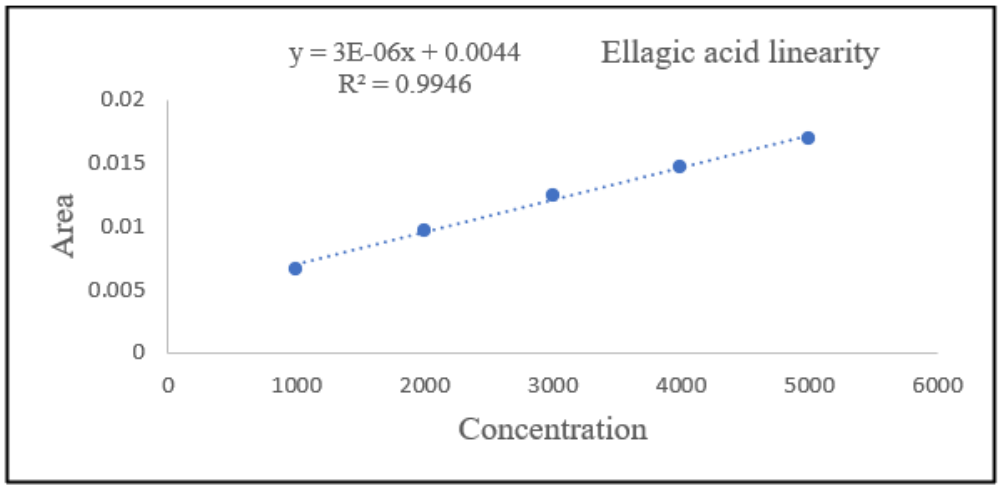

Figure 4. Calibration curve of Ellagic acid.

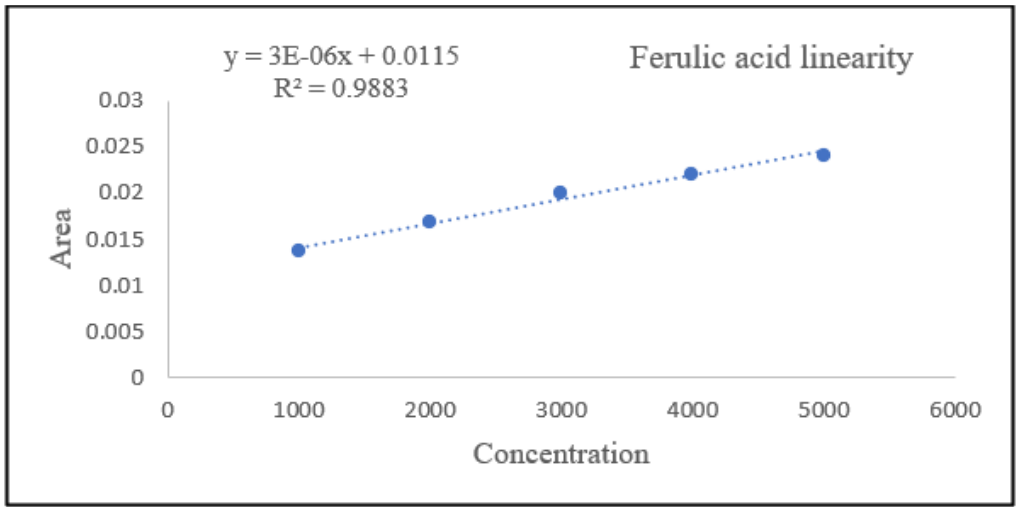

Figure 5. Calibration curve of Ferulic acid.

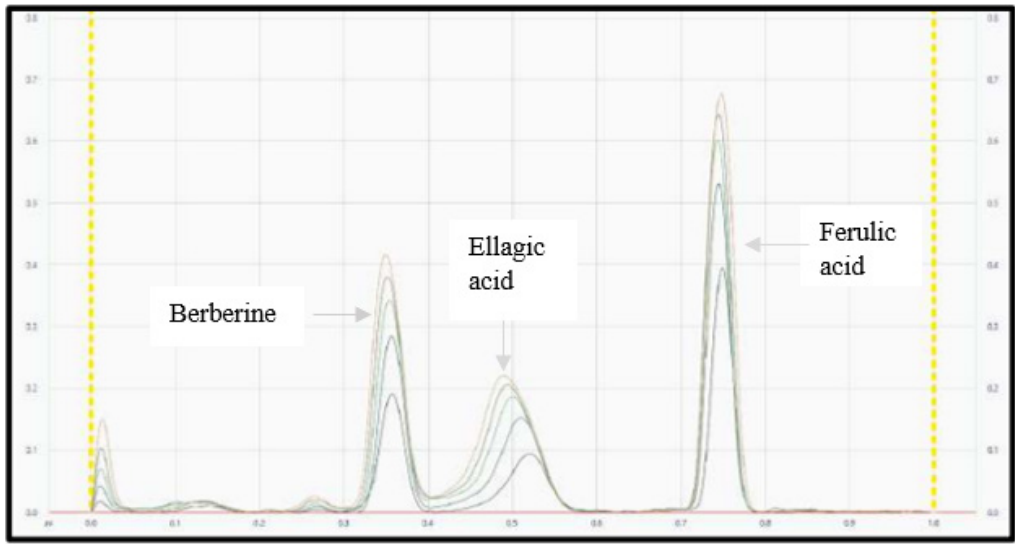

Figure 6. 3D chromatogram of linearity of Berberine, Ellagic acid and Ferulic acid.

3.4 Precision

Intra-day and inter-day precision were calculated with respect to area found SD and %RSD were obtained. As shown in Table 3, the value of %RSD was found to be less than 1%, thereby representing a high level of precision.

3.5 Robustness

For the robustness study, parameters of the optimized method were intentionally changed. By changing (±2) optimized parameters of wavelength and saturation time robustness parameter was evaluated. As shown in Table 4 % RSD was found to be less than 2 and thus the method was found to be robust.

Table 3. Intra-day and Inter-day precision

	Intra-day Precision			Inter-day Precision		
Standards	**Mean Area**	**SD**	**%RSD**	**Mean Area**	**SD**	**%RSD**
Berberine	0.012388	0.000209	1.68	0.011153	0.000152	1.36
Ellagic acid	0.013021	0.00023	1.76	0.009563	0.000175	1.82
Ferulic acid	0.018358	0.000335	1.82	0.017937	0.000307	1.7

Table 4. Robustness

Standards	Wavelength	%RSD	Saturation time	%RSD
Berberine	317	0.34	18	1.3
	319	0.9	20	0.9
	321	1.3	22	1.2
	Wavelength	**%RSD**	**Saturation time**	**%RSD**
Ellagic acid	317	1.5	18	1.8
	319	0.8	20	0.8
	321	1.9	22	1.6
	Wavelength	**%RSD**	**Saturation time**	**%RSD**
Ferulic acid	317	1.3	18	0.4
	319	0.9	20	0.6
	321	1.2	22	1.6

3.6 Specificity

The peak purity of all three markers was assessed by comparing their spectra. A proper regression coefficient was obtained and no interference in the quantification of standards was seen, verifying that the method is specific.

3.7 Accuracy

With the help of the recovered and expected concentrations, the percent recovery was calculated. The average recovery obtained was in the acceptable range (98%-102%) thus proving that the recovery of the proposed method was good (Table 5).

Table 5. Accuracy

Standards	Amount present in sample (ng)	Amount added (ng)	Amount recovered (ng)	Recovery (%)	Average recovery (%)
Berberine	3960	3168	3071	96.95	101.33
	3960	3960	4218	106.52	
	3960	4752	4776	100.51	
Ellagic acid	2650	2120	2017	95.17	98.15
	2650	2650	2612	98.57	
	2650	3180	3202	100.69	
Ferulic acid	1553	1242	1308	105.32	99.82
	1553	1553	1571	101.14	
	1553	1864	1733	92.98	

3.8 Limit of Detection and Quantification

The LOD and LOQ of berberine were reported to be 313 and 948 ng/mL, respectively, while those of ellagic acid were 178 and 540 ng/mL and those of ferulic acid were 266 and 806 ng/ mL, respectively.

3.9 Quantification of Standards

It was essential to properly quantify the presence of standards in the formulation for the study of formulation. With the help of regression equation and area of standards in formulation, the standards were quantified in the given sample and were found as given in Table 6.

Table 6. Quantification of standards

Standards	Amount present in sample (ng/spot)
Berberine	3960
Ellagic acid	2650
Ferulic acid	1553

4. Conclusion

Due to the varied environmental conditions and varied nature of the polyherbal formulation, it is necessary to be sure about the quality of the formulation. So, to ensure the quality of the formulated *Amrtadi churna*, the HPTLC method was developed and validated for berberine, ellagic acid and ferulic acid. The proposed HPTLC method is simple, precise, specific, robust and accurate and can be used for routine analysis of the above-mentioned chemical constituents in any crude drug or formulation.

5. Acknowledgement

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