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QUANTIFICATION OF GALLIC ACID AND ELLAGIC ACID IN ARJUNARISHTA BY VALIDTAED HPTLC DENSITOMETRY

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ABSTRACT

Arjunarishta, also known as Parthadhyarishta, is a polyherbal hydro alcoholic formulation and is advised as a choice of remedy in cardiovascular disorders. A simple, precise and accurate HPTLC method has been established for the determination of quercetin and rutin in Arjunarishta–T and Arjunarishta-M prepared by traditional and modern methods respectively and also in its marketed formulation. The developed HPTLC method was validated in terms of precision, accuracy, LOD, LOQ, specificity, robustness and ruggedness. The amount of gallic acid in Arjunarishta-T, M and its marketed formulation was found to be 0.0332, 0.0331 and 0.0330% w/w respectively while ellagic acid was found to be 0.0361, 0.0360 and 0.0359% w/w respectively. This is the first report for the quantification of gallic acid and ellagic acid in Arjunarishta by HPTLC. Furthermore, no TLC densitometric methods have been reported for the quantification of gallic acid and ellagic acid from Arjunarishta.

INTRODUCTION: Ayurveda, taken from the Sanskrit words *Ayur* meaning life or lifespan and *Veda* meaning knowledge, originated in India but is now practicised throughout the world [1]. There has been increasing interest in Ayurveda, particularly its botanical remedies, because several Indian traditional herbs have been found to produce potent anti-tumour and immunologic effects [2].

Arishtas are an important group of formulations used in Ayurveda. Arjunarishta, also named as Parthadhyarishta, is a poly-herbal hydro-alcoholic formulation. The chief ingredient of Arjunarishta is dried stem bark of *Terminalia arjuna* Roxb. It contains hydrolysable tannins, triterpenoid glycosides (arjunglucoside-1, arjunglucoside-2), and sapogenins as arjunic acid, arjunolic acid, cardenolides, phenolics, flavonoids, phyto-sterols, mineral salt and sugar [3].

It also contains Draksha (fruits of *Vitis vinifera*) and madhuca flowers (flowers of *Bassia longifolia*) which are the rich source of phenolic compounds and possess good antioxidant activity. All these ingredients are used as good dietary sources of antioxidants [4-6].

The major ingredient of Arjunarishta is stem bark of *Terminalia arjuna*. The stem bark of *Terminalia arjuna* has been used for alleviating angina and other cardiovascular conditions [7]. The extract also improved the symptoms of refractory chronic congestive heart failure [8].

Oral administration of *Terminalia arjuna* bark also prevented ischemic reperfusion injury induced oxidative stress and tissue injury of heart in rabbits indicating its beneficial therapeutic effect in ischemic heart disease [9].

The flavonoids which are found present in *Terminalia arjuna* bark have shown antioxidant and lipid lowering effect [10]. HPLC analysis has been carried out for the quantification of some marker compounds for the standardization of Arjunarishta [11]. Furthermore, no validated HPTLC method has been reported for the quantification of gallic acid and ellagic acid from Arjunarishta.

Standardization is an important aspect for establishing the quality and efficacy of Ayurvedic formulations or any poly herbal formulation. Therefore, a proper scientific validation as chromatographic fingerprinting is required for quantification of marker compounds for quality control purposes.

MATERIALS AND METHODS:

Preparation of Arjunarishta-T: The ingredients of Arjunarishta as Arjuna bark (*Terminalia arjuna*), fruits of Draksha (*Vitis vinifera*) and madhuca flowers (*Bassia longifolia*) were procured from local market, Jamnagar. Identification of all the individual plant material was done as per Ayurvedic Pharmacopoeia of India. Authentification of all these ingredients was done in the Department of Botany of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow. Prepared herbarium has been deposited in the CIMAP for future reference.

It was prepared by traditional method as given in The Ayurvedic Formulary of India, part- I, 2000[12]. According to this method, coarsely powdered Arjuna bark (*Terminalia arjuna*), fruits of Draksha (*Vitis vinifera*) and madhuca flowers (*Bassia longifolia*) were placed in polished vessel of brass along with prescribed quantity of water (26L), and allowed to steep overnight.

After overnight steeping this material was warmed at medium flame until the water for decoction reduced to one fourth of the prescribed quantity (6.5 L), then the heating was stopped and liquor was filtered in a cleaned vessel and then 2.5kg of Jaggery (concentrated juice of *Saccharum officinarum*) was added and mixed properly. This sweet filtered liquor was placed in incubator for 15 days at 33°C±1°C after adding Dhataki flowers (*Woodfordia floribunda*). After 15 days, completion of fermentation was confirmed by standard tests [13].

The fermented preparation was filtered with unstarched muslin cloth and kept in cleaned covered vessel for further next seven days. Then, it was poured in clean amber coloured glass bottles previously rinsed with ethyl alcohol, packed and labelled properly.

Preparation of Arjunarishta-M: It was prepared by modern method by carrying slight modifications in the traditional method. Method of preparation was same as followed with Arjunarishta-T, only Dhataki flowers were replaced by Yeast for inducing fermentation [14].

Reagents and Materials: All solvents used were of analytical grade and were purchased from Merck. Gallic acid (purity 98%) was purchased from SD fine, Mumbai. Ellagic acid (purity 97%) was purchased from Yucca Enterprises, Mumbai, India.

HPTLC: Chromatography was performed on 20 x 10 cm HPTLC plates coated with 0.25 mm layers of silica gel 60 F_{254} (Merck, Darmstadt, Germany). Before use the plates were washed with methanol and activated at 110ºC for 5 min. Samples were applied as bands 4 mm wide and 6 mm apart by use of Desaga (Ziegel Wiesen, Germany) AS 30 Win sample applicator equipped with a 100 µL syringe. A constant application rate of 10µL s^{-1} was used. The mobile phase for gallic acid was toluene-ethyl acetate- formic acid- methanol, 6+6+1.2+0.25 (*v/v*), while for ellagic acid was toluene-ethyl acetate-formic acid-methanol, 9+9+3+0.6 (*v/v*), were used for chromatography.

Linear ascending development was performed in a Camag 20cm x 10cm glass twin-trough chamber. Before insertion of the plate the chamber was saturated with mobile phase vapor for 20 min at room temperature (25±2^{o}C) and relative humidity 60 ± 5% by lining the TLC chamber on three sides with filter paper, also placed in the mobile phase. The development distance was 8 cm. After development the TLC plates were dried in a current of air by means of an air dryer.

Densitometric scanning was performed with a Desaga TLC scanner CD 60 in reflectance absorbance mode at λ = 290 nm for gallic acid and λ = 285 nm for ellagic acid controlled by ProQuant software (v1.06; Desaga) resident in the system. The slit dimensions were 4 x 0.02 mm and the scanning speed 100 nm s^{-1}. The radiation source was a deuterium lamp emitting continuous UV radiation between 190-360 nm.

The amounts of the compounds chromatographed were determined from the intensity of diffusely reflected light.

Preparation of Standard Solutions and Calibration Plots:

1. **Preparation of standard solution of gallic acid:** Stock solution of 300 µg mL^{-1} of gallic acid was prepared by dissolving 15 mg of accurately weighed gallic acid in methanol and making the volume of solution up to 50 mL with methanol in volumetric flask. The aliquots (0.5 to 2.5 mL) of stock solutions were transferred to 10 mL volumetric flasks and the volume of each was adjusted to 10 mL with methanol, to obtain standard solutions containing 15, 30, 45, 60 and 75 µg mL^{-1} of gallic acid, respectively.

   10µL each of the standard solutions of gallic acid (150-750 ng $spot^{-1}$) were applied as bands 4 mm wide and 6 mm apart in triplicate on a TLC plate using an automatic sample spotter (AS 30 Win). Linear regression data for the calibration plot are listed in **Table 1**. A good linear relationship between response (peak area) and amount was obtained over the range 150-750 ng/band.

2. **Preparation of standard solution of ellagic acid:** Stock solution of 400 µg mL^{-1} of ellagic acid was prepared by dissolving 20 mg of accurately weighed ellagic acid in methanol and making the volume of solution up to 50 mL with methanol in volumetric flask. The aliquots (0.5 to 2.5 mL) of stock solutions were transferred to 10 mL volumetric flasks and the volume of each was adjusted to 10 mL with methanol, to obtain standard solutions containing 20, 40, 60, 80 and 100µg mL^{-1} of ellagic acid, respectively.

   10µL each of the standard solutions of ellagic acid (200-1000 ng $spot^{-1}$) were applied as bands 4 mm wide and 6 mm apart in triplicate on a TLC plate using an automatic sample spotter (AS 30 Win). Linear regression data for the calibration plot are listed in **Table 1**. A good linear relationship between response (peak area) and amount was obtained over the range 200-1000 ng/band.

Sample Preparation: 1 g (equivalent to 0.94 mL) of each of the test formulation of Arjunarishta as Arjunarishta-T, Arjunarishta-M and its marketed formulation was dried on water bath for half an hour to remove the alcohol. Then, each of the test samples of Arjunarishta was diluted with methanol up to 10 mL and sonicated for 15 min and centrifuged at 3200 rpm to settle down the precipitated sugars. 1 mL of supernatant was passed through 0.45 µm filter (Millipore) and 10µL of each of the test formulation was applied as band on plate for quantification.

Validation of the method: ICH guidelines were followed for the validation of analytical methods developed for precision, repeatability and accuracy[15].

RESULTS AND DISCUSSION:

Selection of the optimum mobile phase: In an attempt to optimize mobile phase, toluene-ethyl acetate-formic acid-methanol mixtures in different proportions were investigated. Use of toluene-ethyl acetate-formic acid-methanol 6+6+1.2+0.25(v/v) resulted in sharp, well defined gallic acid peaks of R_F 0.49±0.02 while solvent system toluene-ethyl acetate-formic acid-methanol 9+9+3+0.6(v/v) resulted in sharp ellagic acid peaks of R_F 0.46±0.02.Well defined bands were obtained only when the chamber was saturated with the mobile phase for 30 min at room temperature before plate development.

Results of Validation of the method: ICH guidelines were followed for the validation of the analytical methods developed for precision, repeatability and accuracy and the results have been shown as follows.

Instrumental precision: Instrumental precision was checked by repeated scanning (n = 6) of the same spot of gallic acid (150 ng $spot^{-1}$) and ellagic acid (200 ng $spot^{-1}$) expressed as relative standard deviation (% RSD) as shown in **Table 1**.

Repeatability: The repeatability of method was affirmed by analysing 150 ng $spot^{-1}$ and 200 ng $spot^{-1}$ individually on TLC plate (n = 6) and expressed as % RSD as shown in **Table 1**.

LOD and LOQ: The limits of detection and quantification were determined by visual evaluation. The detection and quantification limits obtained by

this method for gallic acid were 50 and 150 ng, respectively while for ellagic acid detection and quantification limit were 60 and 200 ng respectively as shown in **Table 1** which indicates that the sensitivity of the method is adequate.

TABLE 1: METHOD VALIDATION PARAMETERS FOR THE QUANTIFICATION OF GALLIC ACID AND ELLAGIC ACID IN ARJUNARISHTA-T, ARJUNARISHTA-M AND ITS MARKETED FORMULATION

Parameter	Gallic acid	Ellagic acid
Instrumental Precision (% RSD, n = 6)	0.54	1.08
Repeatability (% RSD, n = 6)	0.58	1.15
LOD (ng)	50	60
LOQ (ng)	150	200
Linear range (n = 3)	150-750 ng/band	200-1000 ng/band
Correlation coefficient (r)	0.9996	0.9996
Slope	6715.2	2233.3

Intra-day and Inter-day Precision: The intra-day and inter-day precision of the method were estimated by analysing aliquots of standard solution containing 150, 450, 750 ng spot^{-1} and 200, 600, 1000 ng spot^{-1} of ellagic acid on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % RSD in **Table 2** [16]**.**

TABLE 2: INTRA-DAY AND INTER-DAY PRECISION OF THE HPTLC METHOD [(a)]

Marker	Amount [ng/band]	Intra-day precision		Inter-day precision	
		Mean area [AU]	RSD [%]	Mean area [AU]	RSD [%]
Gallic acid	150	2913.9	0.55	2912.1	0.64
	450	4910.6	0.41	4909.5	0.48
	750	6952.9	0.34	6951.2	0.42
Ellagic acid	200	1410.8	1.15	1409.7	1.34
	600	2275.4	0.90	2274.0	1.13
	1000	3197.6	0.60	3196.2	0.69

(a) n = 6

Specificity: The specificity of the method was ascertained by analyzing reference standard and samples. The bands for gallic acid and ellagic acid from Arjunarishta-T, Arjunarishta-M and its marketed formulations were confirmed by comparing the R_F and UV spectra of the separated bands with those from the standard.

Recovery: The pre-analyzed samples of Arjunarishta-T, Arjunarishta-M and its marketed formulation were spiked with an additional 50, 100 and 150% of gallic acid standard and the mixtures were analysed again, in triplicate, by the proposed method, to check recovery of different amounts of gallic acid from the Arjunarishta-T, Arjunarishta-M and its marketed formulation. Recovery was found in between 99.86-100.15% in Arjunarishta-T, 100.06-100.10% in Arjunarishta-M and 99.80-100.13% in the marketed formulation of Arjunarishta as depicted in **Table 3**.

TABLE 3: RESULTS OF RECOVERY STUDY OF GALLIC ACID FROM ARJUNARISHTA-T, M AND ITS MARKETED FORMULATION (n= 3)

Sample	Amount of drug added [%]	Theoretical content [ng]	Recovery [%]	RSD [%]
Arjunarishta-T	50	498	99.86	0.31
	100	664	100.15	0.46
	150	830	100.12	0.43
Arjunarishta-M	50	496	100.06	0.42
	100	662	100.10	0.48
	150	827	100.08	0.39
Marketed Arjunarishta	50	495	100.13	0.65
	100	660	99.95	0.32
	150	825	99.80	0.30

Similarly, the analyzed samples of Arjunarishta-T, M and its marketed formulation were spiked with an additional 50, 100 and 150% of ellagic acid standard and the mixtures were analysed again, in triplicate, by the proposed method, to check the recovery of different amounts of ellagic acid from Arjunarishta-T, M and its marketed formulation. Recovery was found in between 99.82-99.93% in Arjunarishta-T, 99.89-100.23% and 100.04-100.13% in marketed Arjunarishta as shown in **Table 4**.

TABLE 4: RESULTS OF RECOVERY STUDY OF ELLAGIC ACID FROM ARJUNARISHTA-T, M AND ITS MARKETED FORMULATION (N = 3)

Sample	Amount of drug added [%]	Theoretical content [ng]	Recovery [%]	RSD [%]
Arjunarishta-T	50	542	99.82	0.37
	100	722	99.86	0.50
	150	903	99.93	0.28
Arjunarishta-M	50	540	100.19	0.49
	100	720	100.23	0.45
	150	900	99.89	0.51
Marketed Arjunarishta	50	538	100.13	0.47
	100	718	100.05	0.43
	150	898	100.04	0.23

Robustness: Robustness was checked by performing analysis of sample solutions of Arjunarishta as Arjunarishta-T, Arjunarishta-M and its marketed formulation after making small changes to mobile-phase composition toluene-ethyl acetate-formic acid-methanol 6 + 6 + 1.2 + 0.25 and 6.2 + 5.8 + 1.2 + 0.25 (v/v) were tried with two different development distances, 8 and 9 cm for two different amounts of gallic acid, 498 and 830 ng per band from Arjunarishta-T, 496 and 827 ng per band from Arjunarishta-M and 495 and 825 ng per band from its marketed formulation [17].

The low values of % RSD were obtained after introduction of these small changes as shown in **Table 5**. Similarly, robustness was also evaluated by analysing Arjunarishta-T, M and its marketed formulation after making slight changes to mobile-phase composition toluene-ethyl acetate-formic acid 9 + 9 + 3 + 0.6 and 9.2 + 8.8 + 2.9 + 0.6 (v/v) were tried with two different development distances, 8 and 9 cm for two different amounts of ellagic acid, 542 and 903 ng per band from Arjunarishta-T, 540 and 900 ng per band from Arjunarishta-M and 539 and 898 ng per band from its marketed formulation.

Thus, low values of % RSD obtained after introduction of these small changes were indicative of the robustness of the method **(Table 6)**.

TABLE 5: ROBUSTNESS OF THE METHOD FOR GALLIC ACID [A)]

Condition	Arjunarishta-T		Arjunarishta-M		Marketed Arjunarishta	
	Recovery [%][b)]	RSD [%][b)]	Recovery [%][c)]	RSD [%][c)]	Recovery [%][d)]	RSD [%][d)]
Mobile phase composition [e)]						
T-EA-FA-M (6 + 6 + 1.2 + 0.25)	100.12	0.45	100.05	0.53	99.92	0.65
T-EA-FA-M (6.2 + 5.8 + 1.2 + 0.25)	100.09	0.94	100.02	0.79	99.90	0.98
Development distance						
8 cm	100.14	0.51	100.06	0.49	99.95	0.58
9 cm	100.08	0.89	100.03	1.12	99.87	1.09

[a)] n = 6
[b)] Average for two amounts, 498 and 830 ng/band
[c)] Average for two amounts, 496 and 827 ng/band
[d)] Average for two amounts, 495 and 825 ng/band
[e)] T, toluene; EA, ethyl acetate; FA, formic acid; M, methanol

TABLE 6: ROBUSTNESS OF THE METHOD FOR ELLAGIC ACID [A)]

Condition	Arjunarishta-T		Arjunarishta-M		Marketed Arjunarishta	
	Recovery [%][b)]	RSD [%][b)]	Recovery [%][c)]	RSD [%][c)]	Recovery [%][d)]	RSD [%][d)]
Mobile phase composition [e)]						
T-EA-FA-M (9 + 9 + 3 + 0.6)	99.89	0.54	100.15	0.62	100.04	0.76
T-EA-FA-M (9.2 + 8.8 + 2.9 + 0.6)	99.82	0.69	100.11	0.73	100.02	0.56
Development distance						
8 cm	99.93	0.61	100.13	0.56	100.08	0.82
9 cm	99.89	0.77	100.11	0.86	100.05	0.94

[a)] n = 6
[b)] Average for two amounts, 542 and 903 ng/band
[c)] Average for two amounts, 540 and 900 ng/band
[d)] Average for two amounts, 539 and 898 ng/band
[e)] T, toluene; EA, ethyl acetate; FA, formic acid; M, methanol

Ruggedness: Ruggedness is a measure of the reproducibility of a test result under normal, expected operating conditions from instrument to instrument and from analyst to analyst. Ruggedness was tested by analysis of gallic acid 498 and 830 ng per band from Arjunarishta-T, 496 and 827 ng per band from Arjunarishta-M and 495 and 825 ng per band from its marketed formulation; the results are listed in **Table 7** [17]**.** Similarly, ruggedness was measured by performing the analysis of ellagic acid 542 and 903 ng per band from Arjunarishta-T, 540 and 900 ng per band from Arjunarishta-M and 539 and 898 ng per band from its marketed formulation as shown in **Table 8**.

TABLE 7: RUGGEDNESS OF THE METHOD FOR GALLIC ACID [A)]

Variable	Arjunarishta-T		Arjunarishta-M		Marketed Arjunarishta	
	Recovery [%][b)]	RSD [%][b)]	Recovery [%][c)]	RSD [%][c)]	Recovery [%][d)]	RSD [%][d)]
Analyst I	100.10	0.39	100.06	0.71	99.95	0.69
Analyst II	99.98	0.59	100.02	0.61	99.98	0.46

[a)] n = 6
[b)] Average for two amounts, 498 and 830 ng/band
[c)] Average for two amounts, 496 and 827 ng/band
[d)] Average for two amounts, 495 and 825 ng/band

TABLE 8: RUGGEDNESS OF THE METHOD FOR ELLAGIC ACID [A)]

Variable	Arjunarishta-T		Arjunarishta-M		Marketed Arjunarishta	
	Recovery [%][b)]	RSD [%][b)]	Recovery [%][c)]	RSD [%][c)]	Recovery [%][d)]	RSD [%][d)]
Analyst I	99.93	0.64	100.09	0.78	100.01	0.89
Analyst II	100.02	0.48	100.11	0.57	100.04	0.68

[a)] n = 6
[b)] Average for two amounts, 542 and 903 ng/band
[c)] Average for two amounts, 540 and 900 ng/band
[d)] Average for two amounts, 539 and 898 ng/band

Estimation of gallic acid and ellagic acid in Arjunarishta-T, Arjunarishta-M and in its marketed formulation: Gallic acid was found to be 0.0332, 0.0331 and 0.0330 %w/w in Arjunarishta-T, M and its marketed formulation respectively while ellagic acid was found to be 0.0361, 0.0360 and 0.0359 %w/w in Arjunarishta-T, Arjunarishta-M and in marketed Arjunarishta respectively as showed in **Table 9**.

TABLE 9: ESTIMATION OF GALLIC ACID AND ELLAGIC ACID FROM ARJUNARISHTA-T, ARJUNARISHTA-M AND ITS MARKETED FORMULATION BY PROPOSED HPTLC METHOD

Sample	Gallic acid (% w/w)[a)]	Ellagic acid (% w/w)[a)]
Arjunarishta-T	0.0332 ± 0.0002	0.0361 ± 0.0002
Arjunarishta-M	0.0331 ± 0.0003	0.0360 ± 0.0002
Marketed Arjunarishta	0.0330 ± 0.0002	0.0359 ± 0.0003

(a) Mean ± SD, n = 3

The suitability of the method was examined by estimation of gallic acid in Arjunarishta-T, M and its marketed formulation. Bands of R_F 0.49 ± 0.02 were observed in the densitogram for gallic acid standard **(Figure 1)** while the bands of same R_F were observed in the densitogram obtained from the gallic acid isolated from Arjunarishta-T, M and its marketed formulation **(Figure 2)**. Similarly, ellagic acid was also estimated in Arjunarishta-T, M and its marketed formulation. Bands of R_F 0.46 ± 0.02 were observed in the densitogram for ellagic acid standard **(Figure 3)** while the bands of same R_F were observed in the densitogram obtained from ellagic acid isolated from Arjunarishta-T, M and its marketed formulation **(Figure 4)**.

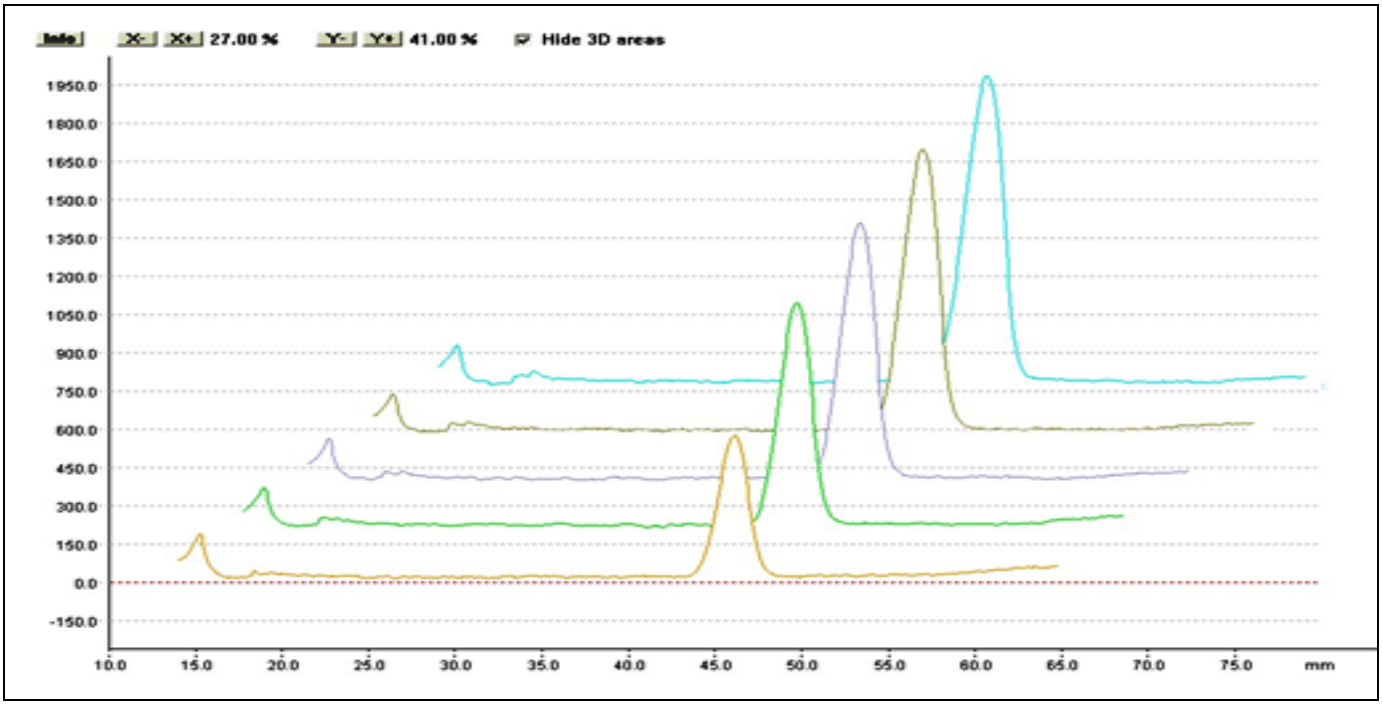

FIG. 1: OVERLAY HPTLC DENSITOGRAM OF GALLIC ACID STANDARD

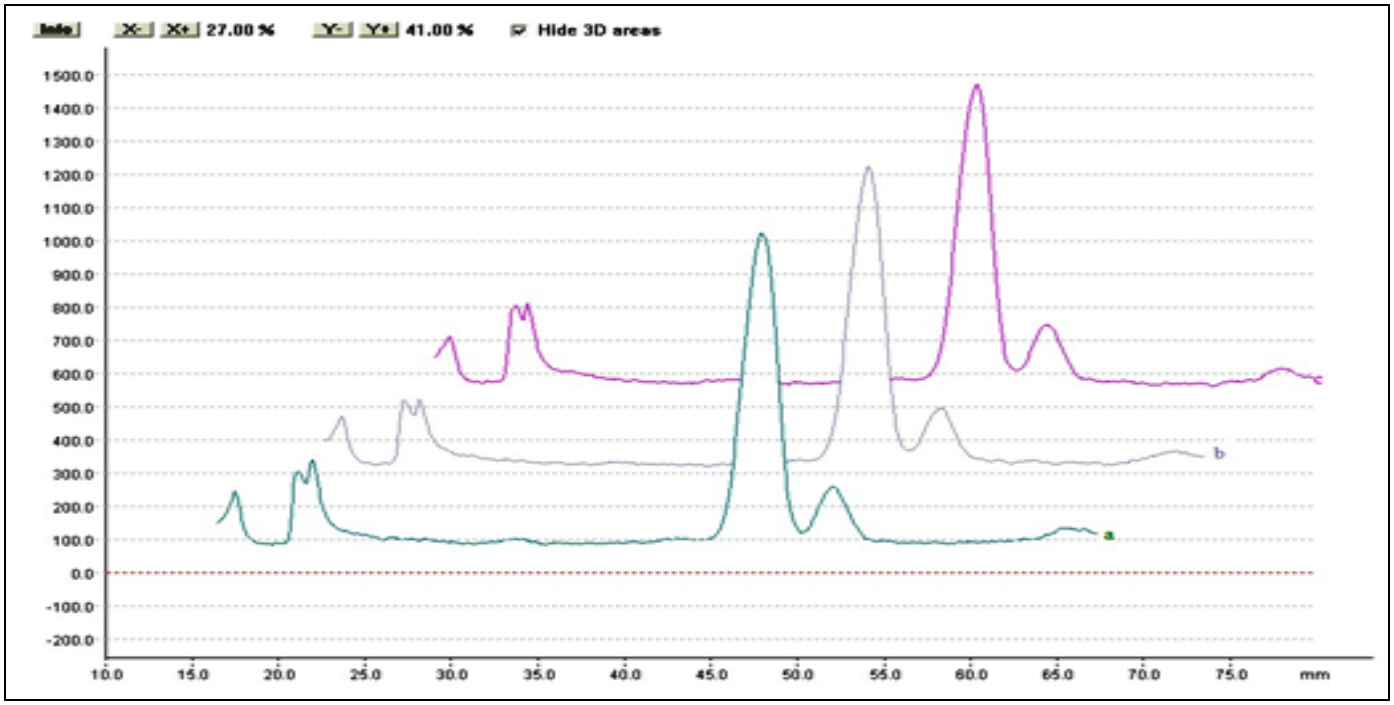

FIG. 2: OVERLAY HPTLC DENSITOGRAM OF GALLIC ACID FROM SAMPLES OF ARJUNARISHTA

a, Arjunarishta-T; b, Arjunarishta-M; c, marketed Arjunarishta

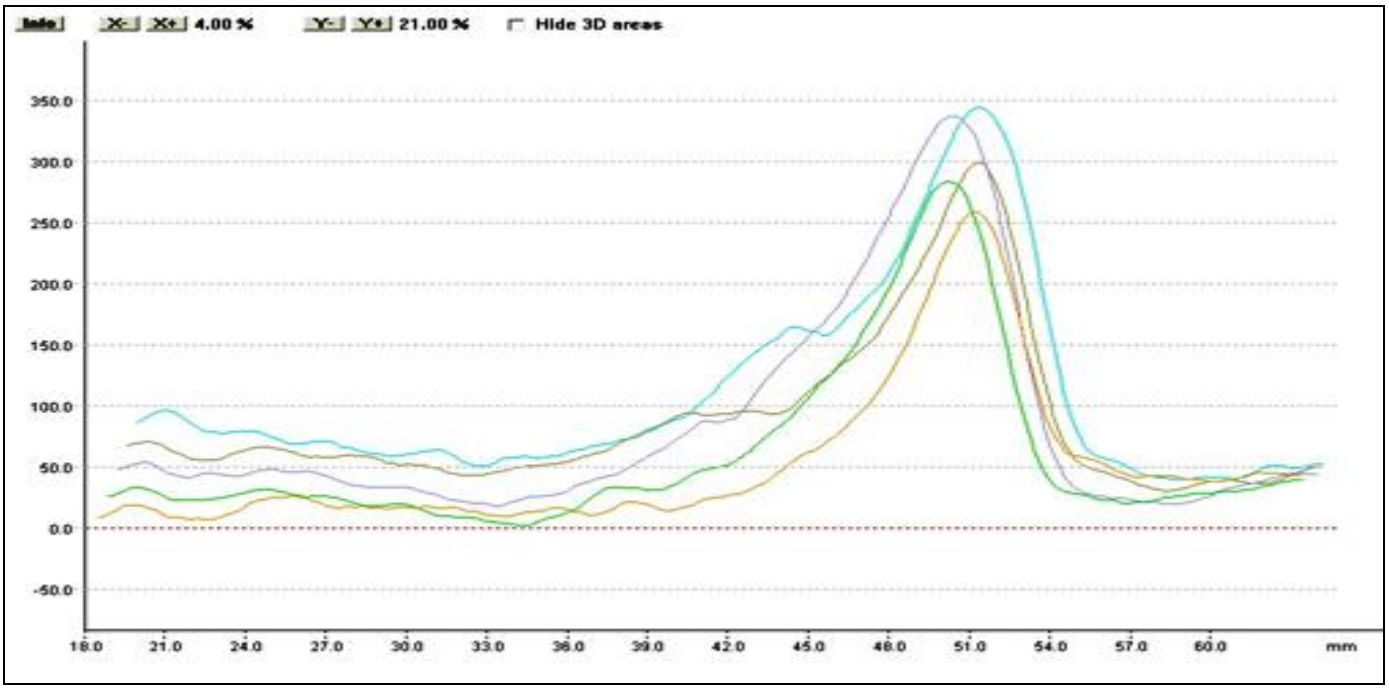

FIG. 3: OVERLAY HPTLC DENSITOGRAM OF ELLAGIC ACID

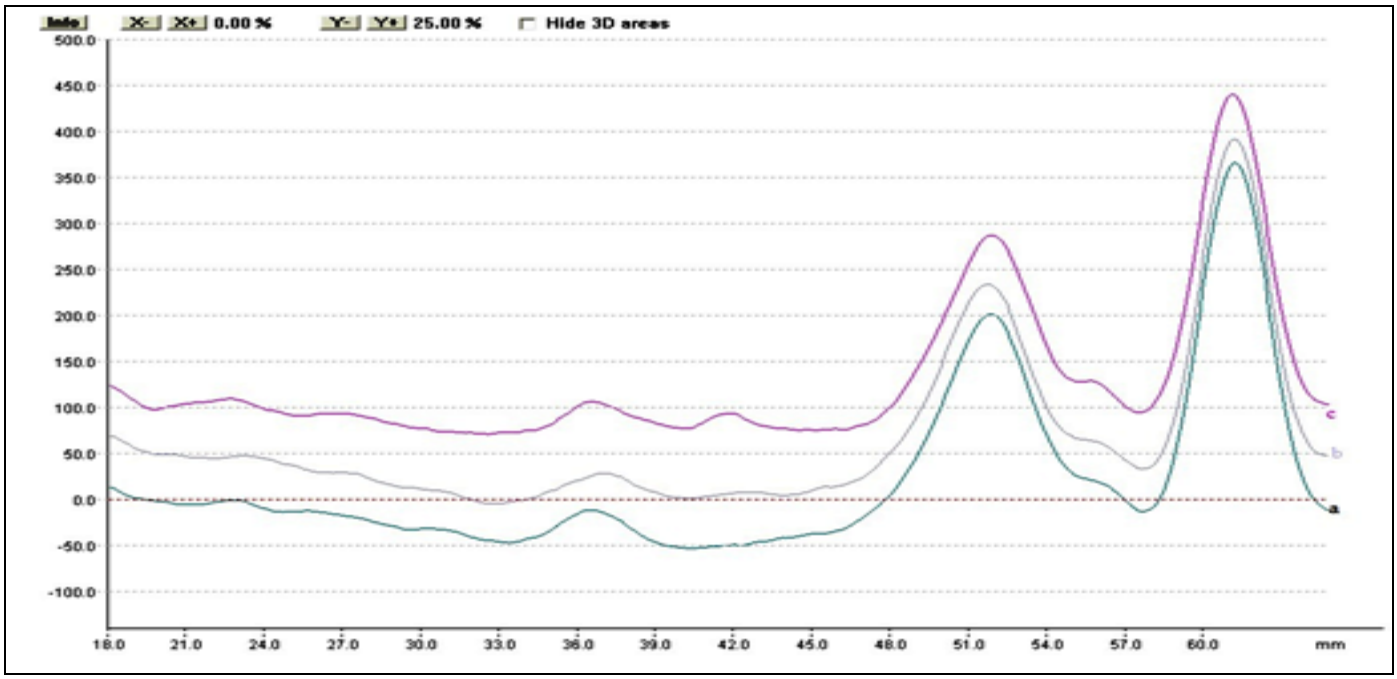

FIG. 4: OVERLAY HPTLC DENSITOGRAM OF ELLAGIC ACID FROM SAMPLES OF ARJUNARISHTA

a, Arjunarishta-T; b, Arjunarishta-M; c, marketed Arjunarishta

CONCLUSION: This HPTLC technique was found to be simple, precise, specific, robust and accurate and could find application in routine quality-control analysis of Ayurvedic formulations.

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