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Development and validation of a new HPTLC Finger printing analytical method for newly formulated Arishta from Ficus religiosa root bark.

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ABSTRACT
Arishtas are considered as unique and valuable therapeutics due to their efficacy, stability and desirable features. Ficus religiosa arishta is a herbal hydro-alcoholic formulation used in the treatment of inflammatory disorders. The self generated alcohol and water present in it accomplish as a media to deliver water and alcohol soluble active components to body. HPTLC studies were carried out using CAMAG HPTLC system equipped with LINOMAT 5 applicator, a TLC scanner, REPROSTAR 3, win Cats software with solvent system toluene: ethyl acetate:formic acid: methanol (14: 10: 2: 1). Various bands were observed in the sample and the phenolic bands with Rf value 0.14, 0.23, 0.28, 0.33, 0.43, 0.53 and 0.60 demonstrated the presence in the formulation from Ficus religiosa. The present study is an attempt to identify marker compounds present in newly formulated arishta from root bark of F. religiosa using high performance thin layer chromatography. The developed HPTLC fingerprint profile can be used for identification of marker compounds. The proposed HPTLC method was found to be simple, precise, accurate etc. and is suitable for the quality control of the formulation.

KEYWORDS: *Ficus religiosa, arishta, HPTLC fingerprinting, marker compounds, validation.*

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I. INTRODUCTION

Medicinal plants are known as the backbone of traditional medicines. India is the largest producer of medicinal plants and has one of the oldest and diverse traditional culture associated with the use of medicinal plants [1, 2] Phytoconstituents from plants play a vital role in primary healthcare as therapeutic remedies. Therefore, many plant species have been used in folklore medicine to treat various ailments [3]. The favorable effects of plants are usually due to secondary metabolites which provide heath promoting properties [4]. A wide variety of active phytoconstituents including flavonoid, polyphenolics, tannins, terpenoids, saponins, plant steroids etc. have been identified from medical plants [5,6] . Medicinal plants are found to be safe and effective for the natural remedy. The therapeutic efficiency of drugs depends greatly on the use of proper and genuine raw materials. Therefore, the quality, safety and efficacy of medicinal plants and herbal products have become a key issue, so that standardization of plants is necessary [7].

Ficus religiosa is a species of fig native to the Indian subcontinent belongs to family Moraceae popularly known as 'bodhi tree' or 'peepal tree' and has got religious and medicinal importance in Indian culture [8, 9]. Because of its contribution in historical events it has an important place in medicinal, mythological and religious systems of India.Almost every part of the tree are rich in phytochemicals and are used in various food and herbal medicinal preparations[10]. Fruits of ficus religiosa are rich in phytochemicals like flavonoids, terpenoids etc. and used to cure respiratory and digestive disorders. Leaves contain flavonoids, tannin etc. which effectively cure diseases like vomiting, antivenom, inflammation etc. Traditionally, barks are used as antibacterial, astringent, antidiarrhoeal, in the treatment of gonorrhea etc. [11, 12]. In the current study, decoction of F. religiosa root bark was taken to prepare arishta which has been produced in accordance with Ayurvedic pharmacopoeia. From herbal formulations separation, identification and estimation of chemical components is very difficult. Literature survey revealed that marker compounds have serious pharmacological properties.

Since herbs are easily available to humans, they have been explored to the maximum for their medicinal properties. And this can be achieved only if the herbal products are evaluated and analyzed using advanced modern techniques [13, 14]. Nowadays, the reporting of various marker profiling have shown to bea useful method for standardizationand quality control of variousherbal materials, especially whenthere is a lack of authenticstandards for the identification ofall active components present inthenatural products[15,16]. For herbal products, Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography

(HPTLC) has become an efficient analytical toolfor their analysis. HPTLC has beenwidely used for the identity andquality of the botanicals because of its versatility, reliability and cost effectiveness. Furthermore, simultaneous analysis of several components in a polyherbal formulation or herbal extracts becomes possible [17]. According to the ICH guidelines in our present study the validation parameters developed were accuracy, precision, specificity and robustness for gallic acid, catechin of the formulation. [18]

HPTLC is a sophisticated instrumental technique based on the principle of separation, resolution and validation process. HPTLC is the simplest separation technique that offers better resolution and estimation of active constituents with reasonable accuracy in shorter time. Due to its simplicity, sensitivity, accuracy, suitability etc. HPTLC has emerged as a preferred analytical tool for fingerprint analysis and quantification of marker compounds in herbal drugs [19, 20]. In the present investigation, we have developed simple, optimized and validated HPTLC method for the identification of marker compounds present in the newly formulated arishta.

II. MATERIALS AND METHODS

Collection and authentication of plant material:-

The root bark of the plant Ficus religiosa was collected from Kozhikode, Kerala, India. The plant was authenticated by Dr. A.K. Pradeep, Associate Professor, Dept. of Botany, Calicut University with specimen No. 88488. The root bark was washed thoroughly with tap water, shade dried and then pulverized to finepowder. It is stored in air tight container for further study.

Extraction

50g of shade dried root bark powder was extracted in 250 ml of ethanol-water mixture (70:30) using Soxhlet apparatus for 72 hours. Repeated extraction was done with same solvents until a clear colorless solvent obtained. The extract was concentrated by evaporating the solvent and the obtained extract was weighed. The dried extract was kept in desiccator prior to analysis.

Preparation of arishta

From the dried extract 25 ml of kashaya was prepared by mixing the extract with boiled and cooled water. Kashaya was strained and poured into the fermentation vessel which was previously rubbed with ghee. 60g of jaggery was dissolved, boiled and filtered using muslin cloth. 25 ml of jaggery syrup was added to the vessel along with 450 ml of water. The prakshepadravya were finely powdered and added to it. At the end, properly washed dhatakipushpa were added. The mouth of the vessel was sealed using clay smeared cloth wound by placing the lid and ensured that it was air tight. The container was kept into a pit in the soil for 30 days at constant temperature.

Table 1: Formulation composition

Ingredients	**Qty.**
Ficusreligiosa (drug) extract	20g
Guda (Jaggery)	60g
Prakshepadravya	
Marica (black pepper)	1.5g
Nagara (dried ginger)	1.5g
Jeeraka (cumin seeds)	1.5g
DhatakiPushpa (woodfordia flower)	1g
Water	450ml

After the specified period lid was removed and the contents examined to ascertain whether the process of fermentation has been completed. The fluid was first decanted and then strained after 2-3 days. After the settling of fine suspended particles, it was strained again and bottled.

Preparation of working standard solution of ellagic acid and catechin

A solution (1 mg/ml) of ellagic acid and catechin 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 ellagic acid and 3–8 ml of catechin) were transferred to 10 ml volumetric flasks and diluted to volume with methanol and applied on TLC plates.

Preparation of sample solution

5.25 g of formulated arishta was extracted with 10 ml of methanol in volumetric flask resulting in a sample concentration of 100 µg/ml and spotted as 10 µl.

HPTLC analysis

10 µl of the above test solutions were loaded as 5 mm band length in the 20 × 10 cm Silica gel $60F_{254}$ TLC plate using a Hamilton syringe and Camag Linomat 5 instruments. The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent iodine vapor) with the mobile phase of Toluene- Ethyl acetate-Formic acid- Methanol (14: 10: 2: 1) and the plate was developed in the same mobile phase up to 90 mm. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (Camag Reprostar 3) and captured the images at Whitelight, UV 254 nm and UV366 nm. Before derivatization, the plate was fixed on scanner stage (Camag TLC Scanner 3) and scanning was done at UV 254 nm. The Peak table, Peak display were noted. Then the developed plate was sprayed with 20% Sodium carbonate solution sprayed and brief dried followed by Folin CIO-calteu reagent and dried at 100 °C in Hot air oven. The plate was photo-documented at Day light using Photo-documentation (Camag Reprostar 3) chamber.

Method validation

- **Precision**
  Six replicates of same concentration of ellagic acid (300 ng/spot) and catechin (300 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 ellagic acid, 300, 500 and 800 ng/spot for catechin.
- **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 ellagic acid and catechin in mixture by standard addition method. To measure the accuracy, known amount of standard solutions of ellagic acid and catechin were spiked to 80, 100 and 120% of a pre-quantified samplesolution 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, formic acid and methanol (14: 10: 2: 1v/v) for ellagic acid and catechin 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 and 20 minutes. Robustness of the method was checked following the same three different con- centration levels as mentioned in precision.

- **Specificity**

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

III. RESULTS

The work attempts to optimize the simultaneous HPTLC profiles of secondary metabolites in newly formulated arishta from Ficus religiosa root bark. The occurrences of metabolites are shown in table No. 2 from the Rf value of the peak. HPTLC outline under UV 254 and 366nm present in fig. 1. The sample was run alongwith standards and was recognized to validate the presence of constituents from the chromatogram after derivatization.

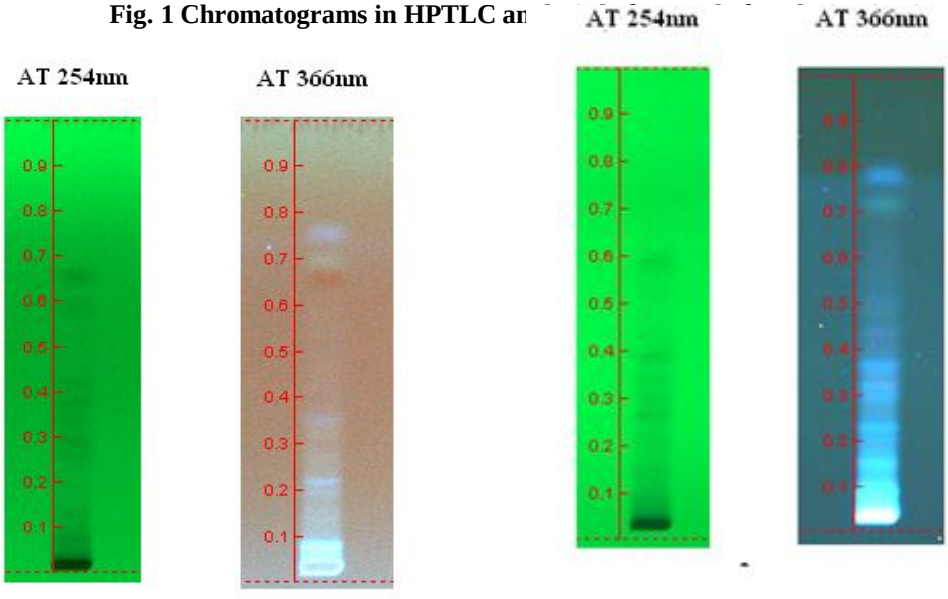
Table No.2 Peak table with Rf values at 254 nm

Peak No.	Rf value	Area (AU)	% Area
1.	0.14	1227.1	13.30
2.	0.23	2300.3	13.76
3.	0.28	2479.1	11.68
4.	0.33	4372.9	15.78
5.	0.35	5219.9	17.60
6.	0.40	5932.3	19.05
7.	0.43	7129.7	10.76
8.	0.53	7505.6	21.81
9.	0.60	9752.1	17.67
10.	0.72	10479.1	18.40
11.	0.77	12375	12.11

Table No.3 Peak table with Rf values at 366 nm

Peak No.	Rf Value	Area(AU)	% Area(AU)
1	0.10	583.6	14.70
2	0.16	2857.5	21.60
3	0.28	3684.3	17.24
4	0.32	6690.0	27.38
5	0.36	7798.8	30.13
6	0.44	9354.8	28.95

Fig. 1 Chromatograms in HPTLC ar



The result from the HPTLC chromatogram for phenolics compound tannins, saponins, alkaloids and steroids can be distinguished at UV 254nm. The newly formulated arishta evidenced 17 spots with corresponding Rf values ranging from 0.10 – 0.77. From table 2, band with Rf value 0.14, 0.28, 0.33, 0.53 and 0.60 revealed the presence of phenolic compounds. Rf value 0.28 validated the presence of catechin in Ficus religiosa root bark. Different types of tannins have been observed at Rf 0.14, 0.33 and 0.53 and 0.60 ± 0.01 revealed the presence of gallic acid derivative. Rf value 0.51± 0.04 validated the presence of ellagic acid which

is a dimeric derivative of gallic acid. In the same way Rf value ranging 0.43 and 0.72 showed the presence of saponins, 0.23± 0.02 showed the presence of steroids and 0.35± 0.03 indicated the presence of alkaloids in the formulated arishta.

Method validation

Table 4. Method validation parameters for HPTLC fingerprinting profile of ellagic acid and catechin

Method property	Ellagic acid	Catechin
R_f	0.53	0.28
Instrumental precision (RSD [%] n = 6)	3.0	3.1
Intra assay precision (RSD [%] n = 6)	2.6	2.4
Intermediate precision (RSD [%] n = 6)	3.4	2.4
Correlation coefficient, r	0.996	0.993
Calibration range [ng]	300–700	300–800
LOD	300	300
LOQ	900	900
Specificity	Specific	Specific
Robustness	Robust	Robust

Linear regression data for the calibration plot as correlation coefficients (r) were found to be 0.996 and 0.993 respectively tabulated in Table 3 and illustrated in Fig. 2.indicated adherence of the method to Beer's law.

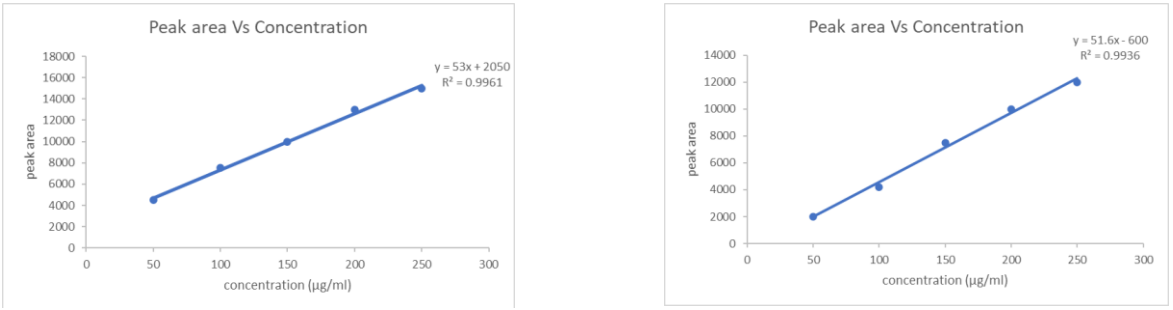


Fig.2 Linearity plot of ellagic acid and catechin

- **Precision**

The spot of ellagic acid (300 ng/spot) and catechin (500 ng/spot) and were expressed as % RSD and was found to be less than 3% as shown in Table 4. 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 5 was found to be precise based on the results obtained in the intra-day and inter-day precision evaluation study.

Amount (ng/spot)	Intra day precision			Inter day precision		
	Mean area	SD	%RSD	Mean Area	SD	%RSD
Ellagic acid						
300	2314.1	1.13	0.049	2184.2	0.52	0.026
500	3478.4	1.07	0.032	3496.6	1.43	0.048
Catechin						
300	1312.5	1.39	0.121	1231.5	1.35	0.112
500	2055.6	1.19	0.063	1992.2	1.18	0.052

Table 5. Intra and inter-day precision of HPTLC

- **Accuracy and recovery studies**

The sample of arishta was spiked with 80, 100 and 120% of ellagic acid and catechin standard and the mixtures were analyzed in triplicate to check the recovery of different amounts of these marker compounds. %RSD for ellagic acid and catechin were found to be and respectively for the formulation of arishta as depicted in Table 6. This shows the accuracy of the method in a desired range.

Table 6 Recovery study of ellagic acid

Level of addition	Amount present in sample (µg)	Amount added (µg)	Recovery (%)	Average recovery (%)	SD	%RSD
80%	100	80	99.73	99.42	0.6101	0.6136
	100	80	98.91			
	100	80	99.64			
100%	100	100	99.29	99.35	0.5099	0.5132
	100	100	98.93			
	100	100	99.84			
120%	100	120	98.72	99.51	0.8929	0.8972
	100	120	99.61			
	100	120	100.21			

Table 7 Recovery study of catechin

Level of addition	Amount present in sample (µg)	Amount added (µg)	Recovery (%)	Average recovery (%)	SD	%RSD
80%	100	80	99.51	99.30	0.4029	0.4057
	100	80	99.66			
	100	80	98.74			
100%	100	100	99.32	99.41	0.4098	0.4122
	100	100	98.86			
	100	100	99.85			
120%	100	120	100.14	99.71	0.3741	0.3751
	100	120	99.23			
	100	120	99.78			

- **Limit of detection and limit of quantification**

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 ellagic acid and catechin and limit of quantification, the lowest amount of analyte that could be quantified was found 900 ng/spot for ellagic acid and catechin as shown in Table 3 which indicates the adequate sensitivity of the method.

- **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**

The peak purity was calculated as per regression (r2). The values for ellagic acid was r2 (start, middle) = 0.9950 and r2 (middle, end) = 0.9943, for catechin r2(start, middle) = 0.9949 and r2(middle, end) = 0.9953. Chromatographic specificity was investigated by comparing the Rf 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.

IV. DISCUSSION

Ficus religiosa root bark arishta is rich in polyphenolic compounds where ellagic acid and catechin are their major tannin-related marker constituents. The development and validation of an efficient analytical method is an integral part of the quality control of the source material so that the safety and efficacy of product or formulation developed is ensured. Simultaneous identification and quantification of these constituents are required for the quality control of herbal formulation prepared from F. religiosa. Hydrolysable tannins are reported responsible for the biological activities of F. religiosa. Arishta is extensively used as an herbal formulation to promote health, immunity and long activity and may have enormous concentration of tannins such as ellagic acid and catechin, and it is pertinent to maintain their quality and purity for safety and efficacy.

V. CONCLUSION

HPTLC fingerprinting analysis can be used as a diagnostic tool for the true identification of the plant. It is observed that, the above HPTLC fingerprinting contains a lot of marker compounds with different Rf values. The proposed HPTLC method seems to be accurate, precise, robust etc. The HPTLC fingerprinting of newly formulated arishta from Ficus religiosa root bark showed higher number of peaks of phenols and tannins in maximum wavelength whereas saponins showed comparatively less number. Very few numbers of peaks were obtained from alkaloids and phytosteroids. In conclusion, the result obtained from qualitative evaluation of HPTLC fingerprint will be helpful in the identification and maintaining quality and efficacy of the arishta.

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