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VALIDATED HPTLC METHOD FOR STANDARDIZATION OF CAFFEIC ACID IN AN AYURVEDIC FORMULATION CONTAINING FICUS SPECIES

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ABSTRACT

A simple, precise, accurate, and specific HPTLC method was developed and validated for estimation of Caffeic acid in methanol extract of a polyherbal formulation 'Nalpamaradi choornam' and its four Ficus species constituents simultaneously. The method employed TLC Aluminium plate pre-coated with silica gel F_{254} as the stationary phase and Toluene: Ethyl Acetate: Formic acid in the ratio 5.0: 5.0: 0.6 (v/v/v) as the mobile phase. The densitometric analysis was carried out in the absorption mode at 325nm (λmax) and fluorescence mode at 366nm. The Rf value was found to be .049 ± 0.02. The above method was validated as per ICH guidelines. Hence this method can be used for routine analysis of Caffeic acid in herbal drug samples.

Keywords *– Polyherbal formulation, Ficus species, Standardization, HPTLC, Nalpamaradi choornam.*

1. INTRODUCTION

Man depends on medicinal plants for their primary health-care. Herbal medicines can be raw plant material, processed plant material as well as medicinal herbal products. Medicinal plants have curative properties due to the presence of various complex chemical substances of varying composition. These chemical compounds are produced in plant cells due to the secondary plant metabolism. Herbal medicines are well accepted in the global market provided it follows the adequate quality standards. WHO emphasizes on quality standards of herbal formulations through scientific validation of single raw drug, for the safety and efficacy. Standardization is an important step for the establishment of a consistent biological activity and chemical profile for assurance of quality. High-performance thin layer chromatography (HPTLC) is one of the sophisticated instrumental techniques for qualitative and quantitative analysis of the herbs and herbal products[1].

'*Nalpamara*' constitute four important plants of Ficus species namely *Ficus racemosa L., Ficus microcarpa L., Ficus religiosa L.,* and *Ficus benghalensis L.* All parts of these trees have extensive applications in medicine. The bark of these species is active ingredient in many Ayurvedic formulations. '*Nalpamaradi choorna*' a mixture of these four is one among them[2]. Their medicinal applications vary from a bitter medicine, for cooling in action, as haemostatic, as a laxative, in improving complexion, in cleaning vagina, and it is useful in *Pitta* and *Kapha*. They are used in the treatment of diabetes, diarrhea, leucorrhoea, menorrhea, nervous disorder, and vaginal diseases, skin

diseases, and piles[3,4]. The bark is antiseptic, antipyretic and vermicide, moreover, the decoction of the bark is used in the treatment of various skin diseases, ulcers, and diabetes and found to be safe and less toxic than aspirin, a commonly consumed anti-inflammatory drug[5]. It is also found to be Cardioprotective[6] and having good free radical scavenging activity[7,8].

The bark of these four Ficus plants is reported to have Tannin, Saponins, Flavonoids, Steroids, Terpenoids, Phenolic acids and Cardiac glycosides as the major class of chemical compounds[9,10]. Caffeic acid, 3,4, dihydroxy cinnamic acid (Fig. 1) is a phenolic acid used in food supplements for boosting athletic performance, exercise-related fatigue, weight loss, cancer, HIV/AIDS, herpes and other conditions (Wikipedia). It has anti-oxidant and anti-inflammatory activity and found to affect the immune system of the body (www.webmd.com).Caffeic acid is reported to be hepatoprotective and having wound healing, antioxidant, anti-inflammatory activities[11]. It also showed the synergistic antioxidant activity of LDL with catechin, and ellagic acid[12]. Caffeic acid is also found to be radioprotective as it diminishes the levels of primary and more complex cytogenic DNA damage in human white blood cells[13]. Its function by suppressing oncogenic disease through the inhibition of cellular topoisomerase I activity is also reported[14]. In another study using HPLC technique free radical scavenging and anticancer activity of Caffeic acid is reported[15].

Despite the widespread application of the constituents of *Nalpamara* in the Indian traditional system of medicine in the treatment of various diseases, little effort has been made to quantify the active component in these plants. The simplicity of the sample preparation and the possibility of analyzing several samples simultaneously in a short time make HPTLC the method of choice. Hence a densitometric HPTLC method has been developed in the present work for quantitation of caffeic acid from the methanol extract of *Nalpamaradi choorna* and dried stem bark of *F.benghalensis, F.racemosa, F.religiosa* and *F.microcarpa*. This method was found to be suitable for the qualitative and quantitative analysis of plant materials.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Caffeic acid was purchased from Sigma-Aldrich, Mumbai. Toluene, Ethyl Acetate, Methanol, and Formic acid used in the present work were of HPLC grade and were procured from Merck Chemicals, Mumbai, India. The fingerprinting and quantification were done using pre-coated silica gel 60 F_{254} TLC plates (E.Merk, Germany) with aluminium sheet support. The HPTLC system was of CAMAG, Switzerland.

2.2 Preparation of Extract

2.2.1 Authentication of the Plant

Fresh barks of *F.benghalensis (Specimen No. 2209 of H. Santapau), F. religiosa (Specimen No. B 1197 of T.S.Sabnis), F.racemosa (Specimen No. 3783 of H. Santapau)* and *F. microcarpa (Specimen No. NI.-2886 of N.A. Irani)* were collected and were authenticated by Blatter Herbarium, St. Xavier's College, Mumbai. They were dried under shade and homogenized to a fine powder separately and stored at 25°C.

2.2.2 Extraction of Material

Preparation of sample solutions were optimized to achieve good fingerprinting and efficient extraction of the marker compound. Two gram each of the powdered drug and the formulations (Procured from market -A and B, and prepared in the laboratory by mixing equal amounts of the four constituents-C) were extracted with methanol (10 ml × 4) under reflux on a water bath. It was filtered through Whatman I filter paper, filtrates were combined, concentrated under vacuum and the volume was made up to 10.0 ml in a volumetric flask. Each of the solutions containing 200 μg/μL of the drug was spotted for the assay.

2.2.3 Instrumentation and Chromatographic Conditions

Chromatographic separation of samples was performed on pre-coated silica gel 60 F254 plate 20×10 cm with 250 µm layer thickness E. Merck, Germany. The plates were activated at 110°C for 5 minutes prior to chromatography. A constant application rate of 0.1µL/spot was employed, and the space between two bands was 10.0mm. The bands were of 6.0 mm width at a distance of 8.0 mm from lower edge and 15.0 mm from the sides. The slit dimensions were 5mm×0.45mm micro and scanning speed 20mm/s with data 100µm/step resolution. The samples were applied under continuous drying stream of nitrogen gas at a constant application speed using CAMAG 100µl sample syringe with an automatic TLC sample applicator Linomat V. The mobile phase consisted of Toluene: Ethyl acetate: Formic acid 5:5:0.6; (v/v/v) and 20.0 ml of it was used for the chromatography run. Linear ascending development was carried out in a 20×10 cm twin trough glass chamber CAMAG, Switzerland saturated with the mobile phase for 15 minutes at room temperature(25°C±2). Each chromatogram was developed over a distance of 8.0cm followed by drying in a stream of air with the help of a hair drier. Densitometric scanning was performed using CAMAG TLC scanner 3 in the reflectance mode at 325nm. The source of radiation used was Deuterium lamp emitting a continuous UV spectrum between 200 and 400 nm. The data processing was done with the software platform win-CATS (CAMAG).

2.2.4. Standard Solution and Calibration Curve

Caffeic acid (10.0mg) was weighed accurately and dissolved in 10.0 ml methanol in a standard measuring flask. This stock solution was diluted further to obtain a concentration of 0.1µg/ µl and used as the standard solution for the HPTLC analysis.

Calibration was done by applying aliquots of 1.0-8.0µL of the standard working solution of Caffeic acid by a micro litre syringe with the help of automatic sample applicator Linomat V on TLC plate, which gives a concentration range of 100-800 ng/spot of Caffeic acid. The plates were developed on a previously described mobile phase. The peak areas plotted against the corresponding concentrations to obtain the calibration graphs.

2.4 Detection and Quantification

The solvent system consisting of Toluene-Ethyl Acetate-Formic acid (5:5:0.6, v/v/v) gave good resolution for all the samples under study. After sample application the plates were developed in a CAMAG twin through chamber pre-saturated with the mobile phase. After development the plates were dried with a hair drier and observed under Camag UV cabinet (254 and 366 nm- Fig.2). The spectral scanning of the developed plates was done using Camag TLC scanner III equipped with win-CATS-V software (Camag). Quantitative analysis of the compound was done by scanning the plates at 325nm. The identification of Caffeic acid was confirmed by superimposing the UV spectra of the samples and standard within the same Rf (0.49) window. (Fig.2)

An HPTLC densitometric analysis of Caffeic acid was also performed for the development of characteristic fingerprint profile which may be used as marker for quality evaluation and standardization of the drug[16] (Fig.3).

2.5 Validation of HPTLC Method

The developed HPTLC method was validated as per International Conference on Harmonization guidelines (CPMP/ICH/281/95 and CPMP/ICH/381/95) for linearity, precision, repeatability, limit of detection, limit of quantification and accuracy.

2.5.1 Linearity and Construction of Calibration Curve

The linearity of the method was studied by constructing a calibration curve at eight concentration levels. Aliquots of the standard working solution of Caffeic acid (1.0-8.0µl) were applied to the plate to obtain a concentration range of 0.10- 0.80 µg per spot. The plate was developed in a twin trough glass chamber containing 20.0 ml of the solvent system saturated for 15.0 minutes. The length of run was 80.0 mm. The developed plate was dried in a stream of air with the help of hair dryer. Scanning was done in UV light at 325 nm. The slit dimensions were kept at 5×0.30 micro at scanning speed at a data resolution of 10 nm/ step. After scanning peak areas were noted and plotted against the corresponding concentration of Caffeic acid spotted. Fig.4.

2.5.2 Precision and Reproducibility

ICH guidelines were followed for the validation of analytical method developed for precision, repeatability and acccuracy. Instrumental precision was measured by replicate(n = 10) application of the same Caffeic acid solution . Variability of the method was studied by analyzing aliquots of the solution on the same day (intra-day precision) and different days (inter-day precision), and the results were expressed as % R.S.D.

2.5.3 Limit of Detection and Limit of Quantification

The limit of detection is the lowest limit at which the analyte of interest is detected and determined by a signal to noise ratioof 3:1 and limit of quantification are the lowest limit at which the analyte of interest is quantified with suitable precision and accuracy, and it is determined by a signal to noise ratio10:1.

2.5.4 Recovery

The accuracy of the method was assessed by performing recovery study at three different levels (80%, 100% and 120% addition of Caffeic acid). The known amounts of Caffeic acid standard were added by spiking. The percent recovery was calculated using the following equation:

$$Percent\ Recovery = \frac{Observed\ Peak\ area}{Expected\ Peak\ area} \times 100$$

Average value of percent recovery for Caffeic acid was also calculated.

2.5.5 Specificity

Specificity of the method was ascertained by analyzing the peak purity profile of the standard compound and samples. The spot for Caffeic acid in the sample was confirmed by comparing the R_f value and spectra of the spot with that of the sample. The peak purity of Caffeic acid was assessed by comparing the spectra at three different levels, ie, peak start, peak middle and peak end position of the band.

2.6 Method Applicability

2.6.1 Estimation of Caffeic Acid in the Methanol Extracts

To estimate the content of Caffeic acid in the herbal extracts, the sample solutions were prepared by refluxing method as described earlier. The standard and the sample solutions (10.0µL) in duplicate were applied on the TLC plate followed by development and scanning. The analysis was repeated six times to study the possibility of interference from the other compounds of the extract on analysis. The superimposable peaks observed (Rf 0.49 corresponding to Caffeic acid) confirmed the presence of Caffeic acid in the samples. (Fig. 5).

3. RESULTS AND DISCUSSION

For the quality assessment and species authentication of traditional medicine, chromatographic fingerprint analysis has been a rational and feasible approach. Chromatographic technique is utilized for constructing specific fingerprint pattern for identification of the medicinal plant. This developed fingerprint pattern can be utilized to detect the presence or absence of marker compounds of interest as well as the ratio of all detectable analytes. High performance thin layer chromatography (HPTLC) is an effective tool for comprehensive quality evaluation of herbal product due to its simplicity, low cost and requirement. It can analyze several samples simultaneously in a short time. The simplicity of the sample preparation also makes HPTLC the method of choice. The unique feature of the picture like image of HPTLC coupled with digital scanning profile is definitely attractive to the herbal analysts to construct the chromatographic fingerprint of herbal samples.Two closly related herbal medicines also can be identified and differenciated by applying the information provided by HPTLC technique.

In the present study, HPTLC fingerprint pattern for the methanol extracts of the four Ficus species and the formulation have been evolved under the identical chromatographic conditions. Quantitation of Caffeic acid standard was done using silica gel F_{254} HPTLC pre-coated plates with mobile phase Toluene- Ethyl acetate- Formic acid (5:5:0.6 v/v/v). The three- dimensional pattern of the standard and the samples demonstrated superimposable peaks with R_f value of 0.49±0.02, which confirmed the presence of Caffeic acid in all the samples.

3.1 Densitometric Quantification of Caffeic Acid using HPTLC

HPTLC densitometric quantification of *F. benghalensis, and F.religiosa,* have been reported using Caffeic acid as marker compound[17]. But F. microcarpa and F. racemosa are not reported . A detailed chemical and analytical screening is done on the bark extract of *F. benghalensis* using HPTLC and an anti-diabetic drug stigmat-5en-3β-O-p-glucoside was identified as a bio-marker[18]. Densitometric quantification of Stigmasterol and Lupeol in F. religiosa by HPTLC was also reported[19]. Some research groups have done the phytochemical evaluation of various parts of Ficus species plants using HPTLC but not the bark[20-22].

There is no report of quatification of Caffeic acid in *Nalpamaradi choorna* and its four Ficus constituents by HPTLC. Hence we have developed a simple and precise method for the quantification of the marker compound. The HPTLC detection parameters were optimized for the quantitative analysis. The mobile phase developed, consisting of Toluene: Ethyl acetate: Formic acid (5:5:0.6), demonstrated better, sharp, and well defined peak resolution. The spot at 0.49 was identified as Caffeic acid in comparison with the chromatogram of the standard compound. The chamber saturation time was found to be 15 minutes prior to the development for better reproducibility.

The TLC plate was visualized at 254nm and 366 nm using CAMAG visualization chamber and photographs were taken. The scanning of the plate was done at 325nm, the wavelength at which maximum absorption takes place. The identity of Caffeic acid band in the sample chromatogram was confirmed in comparison with the densitogram of the standard solution and by comparing the retention factor of Caffeic acid from the sample and standard solutions. The peak corresponding to Caffeic acid from the sample solution had same retention factor as that from Caffeic acid standard (R_f 0.49) (Fig. 5).

In the linearity study, the response was found to be a linear function of the amount applied in the range 100-800 ng/spot. The correlation coefficient obtained was 0.9965.The peak area (y) is proportional to the concentration of Caffeic acid (x) following the regression equation y=223.645+8.489*X and sdv=4.55%. The limit of detection (LOD) value for Caffeic acid was found to be 92.28 ng, and limit of quantification (LOQ) value was 262.69 ng. Table.3.These values are indicative of the sensitivity of the method.

The percentage recovery was in the range of 96.06% to 98.9% measured at three different concentration levels. The average recorded as 97.5% (Table 1). This indicates the accuracy of the method.

The measurement of peak area at 10 different concentration levels showed %CV 2.214 for inter-day and 2.347% for intra-day, which suggested very good precision and reproducibility of the method. Table 2.

The HPTLC densitometry method was validated regarding precision, repeatability, and accuracy or recovery. The results of the validation studies are summarized in Table 3.

Among the four Ficus barks, caffeic acid content was found to be maximum in F. microcarpa (0.0637%) followed by F.racemosa (0.0418%). F. religiosa contain comparatively lesser amount (0.0267%) and the least is in F. benghalensis (0.0125%). The Caffeic acid content in the three formulations was found to 0.129%, 0.107% and 0.105 % w/w basis respectively Table 4.

Nalpamaradi Choorna and its Ficus species constituents were standardized for the first time with Caffeic acid. The method of standardization of F. benghalensis and F. religiosa using HPTLC technique is explained in the 'The Quality Standards of Medicinal Plants. The amounts of Caffeic acid is expected to be in the range of 0.0025-0.0077% (w/w). The higher concentration observed (0.0125% and 0.0267%) could be attributed to the difference in the method of preparation of sample solutions. The present method of preparation

of solution is found to be much simpler and efficient. Standardization of F. racemosa has been reported but not using Cafffeic acid (ICMR 2005). There is no report on standardization of F. microcarpa.

The amount of Caffeic acid in the formulations A, B, and C was found to be varying from each other. The amount of Caffeic acid in B and C the laboratory prepared one were comparable. The content is maximum in formulation A (0.128%). This could be due to the geographical, seasonal, or sample (combination) variations. But it's a matter of interest that, in all the three samples studied the amount of Caffeic acid is much greater than the average mass present in the constituents. This could be due to synergism of all the constituents. This is in support of the report on synergistic antioxidant and antimicrobial activity exhibited by this formulation[23]. This also justifies the traditional usage of 'Nalpamaram' in the combination targeted therapies.

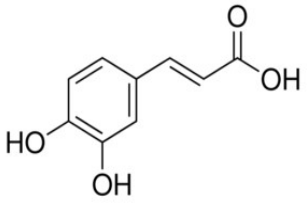

Fig. 1: Structure of Caffeic acid-3,4, dihydroxy cinnamic acid

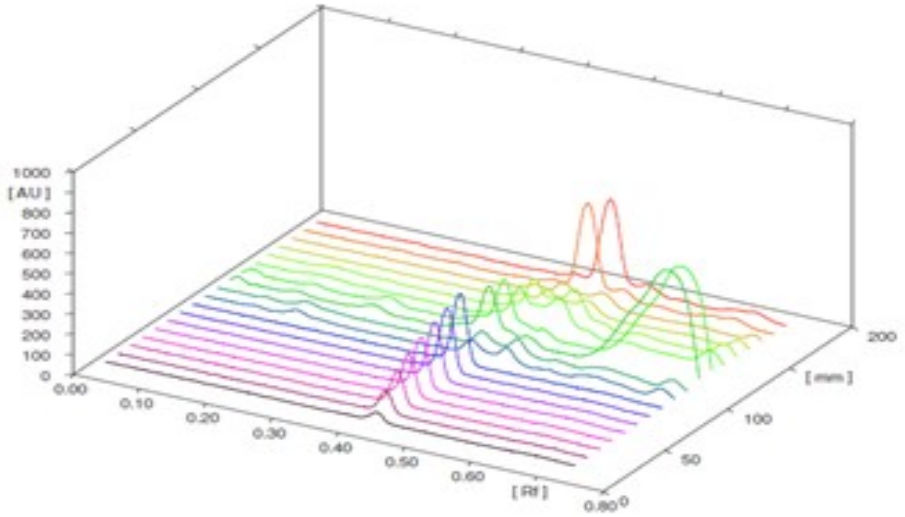

Fig.2: UV spectrum of Standard (Caffeic acid) and raw materials at 325 nm.
Tracks 1-8: Caffeic acid, 9,10- *F.benghalensis*, 11,12- F. racemosa, 13,14- *microcarpa.,* 15,16- *F. religiosa, 17,18- Formulation A.*

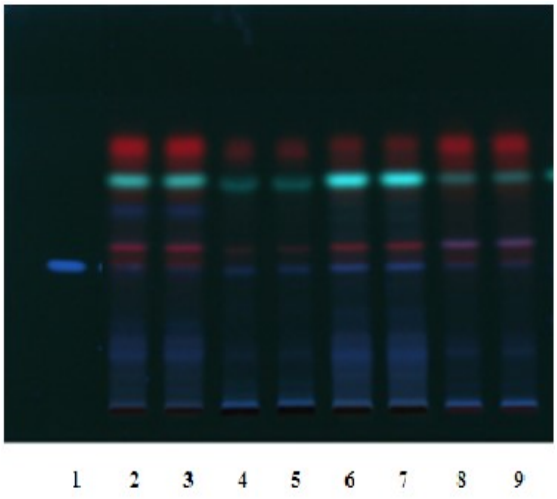

Fig.3. HPTLC Fingerprint profile of Standard (Caffeic acid) and raw material at 366 nm. 1.Caffeic acid, 2,3- *F.benghalensis*, 4, 5- F. racemosa, 6,7- *microcarpa.,* 8,9- *F. religiosa*

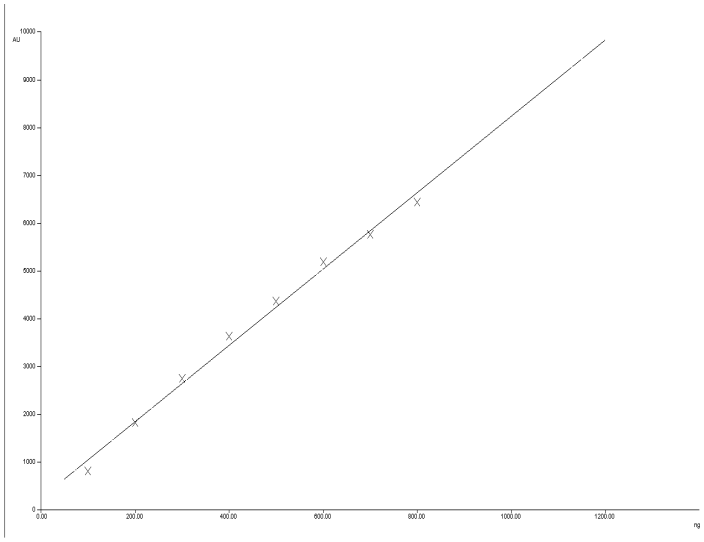

Fig. 4: Calibration curve for standard Caffeic acid (100- 800 ng/spot)

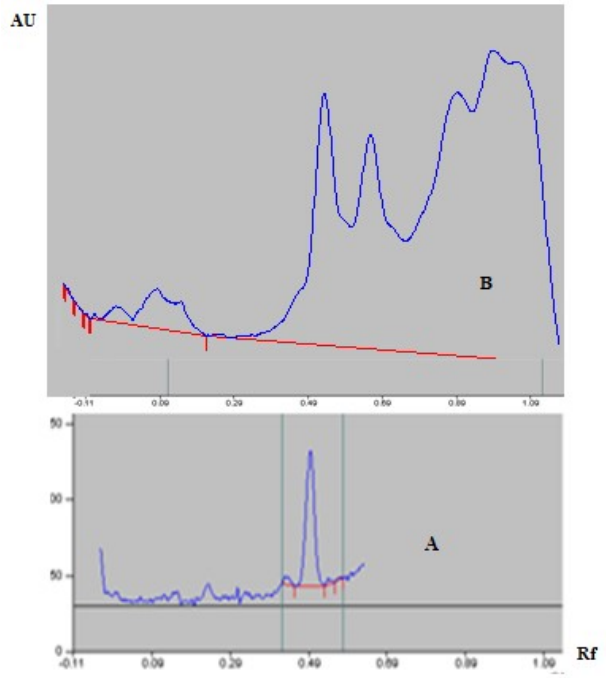

Fig. 5: HPTLC densitometric scan of Formulation C at 325 nm
A: Caffeic acid B: Methanol extract of formulation C

Table 1: Results of Precision studies

Result via area Substance	Rf	X(average)	CV%	n	Remark
Caffeic acid	0.49	7738.997	2.214	10	Inter-day
Caffeic acid	0.48	7791.141	2.347	10	Intra-day

Table 2: Recovery studies of the formulation by HPTLC method

% Level	Concentration of the drug added µg /spot	Concentration of drug via area		% Recovery	Mean
		Added	Found		
80.0	0.32	6729	6653	98.90	97.50%
100.0	0.40	7367	7193	97.67	
120.0	0.48	7526	7825	96.06	

Table 3: Summary of Validation Parameters

S. No.	Parameter	Caffeic acid
1	Precision (% CV, n = 10)	2.214
2	Repeatability (% CV, n = 10)	2.194
3	Accuracy (average % recovery)	97.5%
4	Limit of detection (ng)	92.28
5	Limit of quantification (ng)	262.69
6	Specificity	Specific
7	Linearity (Correlation coefficient) R^2	Y=8.489X +223.645 (R^2 = 0.9965)
8	Range (ng/spot)	100-800

Table 4: % w/w of Caffeic acid in Ficus samples and Formulation (n=6)

No	Sample	Amount of Caffeic Acid (% w/w)	Amount of Caffeic Acid Reported % w/w*	Reference
1	*F. benghalensis*	0.0125 ±0.004	0.0032-0.0077	ICMR, 2008
2	*F. racemosa*	0.0418±0.012	--	
3	*F. religiosa*	0.0267±0.008	0.0026-0.0074	ICMR, 2008
4	*F.microcarpa*	0.0637±0.013	--	--
5	Formulation A	0.128±0.015	--	--
6	Formulation B	0.107±0.007	--	--
7	Formulation C	0.105±0.013	--	--

**The amount depends on the method of extraction*.

4. CONCLUSION

The activity of a plant extract is always influenced by the quantity of active principles present in the extract. Since Caffeic acid can be used in the treatment of various diseases, it was very essential to develop a method for standardization, so as to optimize its quantity in the herbal formulations.

Hence a rapid, simple, precise, accurate and specific HPTLC method has been developed and validated for the quantification of Caffeic acid in the stem bark of *F.benghalensis, F.racemosa, F.microcarpa, F.religiosa* and *Nalpamaradi choorna*, a formulation that constitute these four Ficus species.

Caffeic acid content in the formulation was found to be in higher amount as compared to that of the constituents. Among the constituents, *F. microcarpa* contain the maximum amount followed by *F. racemosa,* and *F.religiosa*, while the least in *F.benghalensis*. The medicinal properties of these Ficus species plants could be attributed to the biological activities of Caffeic acid also. The present investigation reveals that it is judicious to mix these four Ficus species in the formulation of *Nalpamaradi choorna*. Therefore the traditional Ayurvedic medicine *Nalpamaradi choorna* may have potential application in the combination targeted therapies for various neurodegenerative diseases, dermatological problems, as well as radioprotective medicines with minimum side effects.

5. ACKNOWLEDGMENT

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6. CONFLICT OF INTERESTS

The author declare that there is no conflict of interests regarding the publication of this paper.

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