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# Development and validation of a simple HPTLC method for the analysis of (-)-epicatechin simultaneously in *Averrhoa carmbola* L. and *Acacia nilotica subsp indica* L. bark extracts

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## ABSTRACT

*A sensitive and accurate high-performance thin-layer chromatographic method has been developed, validated and used for quantification of (-)-epicatechin in ethyl acetate fractionate of dried bark powder of Averrhoa carambola L. (AC) and Acacia nilotica subsp indica L. (AN). Chromatographic separation was carried out using silica gel plates with cyclohexane-ethyl acetate-formic acid, 4.0:6.0:1.0 (v/v/v) as a mobile phase. Detection and quantification were performed by densitometry, with a deuterium lamp, at 280 nm. The response to (-)-epicatechin reference standard was linear in the concentration range of 200-1600 ng per band. The method was validated for precision, repeatability and accuracy. Intra-day and inter-day relative standard deviation was ≤2%. Instrumental precision and repeatability of the method were found to be 1.02 and 1.59 respectively (% CV). The accuracy was checked by studying recovery at three levels; average recovery was 90.97% and 91.15% for AC and AN respectively. The method proposed for quantitative monitoring of (-)-epicatechin in AC and AN is rapid, simple and accurate and can be used for routine quality testing.*

**Keywords:** *Averrhoa carambola* L., *Acacia nilotica subsp indica* L., (-)-epicatechin, HPTLC, validation.

## INTRODUCTION

*Acacia nilotica* Linn. indigenously known as Babul or Kikar is a proverbial, medium sized tree and is broadly scattered in tropical and subtropical countries. The plant parts of *Acacia nilotica* Linn (AN) has been widely reported to have therapeutic uses arising from its wide spread folkloric and traditional uses. However, hardly any work has been carried out on the *Acacia* species toward documenting its ethno medicinal uses and establishing its phytochemical parameters [1].

Averrhoa carambola L. (Oxalidaceae), commonly known as Kamrakh or Golden star is a unique creation of nature grown widely in the tropics and the warmer areas of subtropics. It is considered as one of the best Indian cooling medicines with many therapeutic activities. These properties are believed to be mediated by different phytochemicals found in plant, acting singly or in combination [2]. Both these plants contain variety of bioactive components such as phenolic acids [2,4], alkaloids [4,5], tannins [4,5,6] and flavonoids

[4,5,7] which are responsible for numerous biological and pharmacological properties. The plants are considered to be hypoglycemic [8,9]. Bark of AN is reported to treat diarrhea [10]. The plants have been shown to exhibit antibacterial, anti-inflammatory, antihypertensive, antispasmodic activities, and antioxidant activity [1,2]. The phytochemical screening of these medicinally useful plants is hardly available. Literature survey reveals work done on separation, purification of phytochemicals from leaves and fruit of these plants. However no HPTLC method has been reported in literature for quantitation of (-)-epicatechin from *Averrhoa carambola* Linn. and *Acacia nilotica subsp indica* Linn. bark powders.

## MATERIALS AND METHODS

**Reagents, Chemical and Standards:** All reagents were of analytical grade. Cyclohexane, methanol, ethyl acetate and formic acid were obtained from Qualigens Fine Chemicals (Mumbai, India). (-)-epicatechin standard (purity ≥90%) was obtained from Sigma-Aldrich Chemie (Aldrich Division; Steinheim, Germany).

**Plant material:** *Averrhoa carambola* Linn was collected from Keshavshrusti (Bhayander), Mumbai and *Acacia nilotica subsp indica* Linn from Warnanagar, Kolhapur. A herbarium of AC and AN, was authenticated by Botanical Survey of India (BSI), Pune, India. The bark of AC and AN was washed with water to remove dust and other extraneous matter. The bark of AC and AN was then shade dried, finely powdered using an electric mixer-grinder and powder was passed through a BSS mesh No. 85 sieve and stored in an airtight container, at room temperature (28 ± 2$^{o}$C). The container was labeled with details, such as date of collection, region of collection and period of collection.

**Preparation of stock and working standard solutions of (-)-epicatechin:** A stock solution (1000.0 µg mL$^{-1}$) of (-)-epicatechin was prepared by transferring 10.0 mg (-)-epicatechin standard in a 10 mL volumetric flask having 5mL of methanol and sonicating for 5 min. The contents of the flask were then diluted up to the mark with methanol. This stock solution (1 mL) was diluted to 10 mL with methanol to give the working standard solution (100.0 µg mL$^{-1}$). Varying volumes of stock solution (2, 4, 6, 8, 12, 14, 16 µL) were spotted on TLC plate to obtain concentrations in the range of 200-1600 ng per band.

**Sample preparation of AC:** Accurately weighed about 3.0 g of dried bark powder of *Averrhoa carambola* Linn. was refluxed at 70$^{o}$C for 2 h with 105 mL of 70% ethanol in a round bottom flask. The contents of the flask were filtered through Whatman paper no.41 and evaporated to dryness using rotary evaporator. Dried extract yielded 0.5 g of 70% ethanol extract from reflux, was then dissolved in water and partitioned 8 times with 50 mL of ethyl acetate. All the extracts were finally pooled and evaporated to dryness using the rotary evaporator which yielded approximately 57 mg of ethyl acetate (EtOAc) extract. Accurately weighed 50 mg of ethyl acetate fractionate extract of AC was transferred into 10 mL volumetric flask containing 5 mL methanol, sonicated for 5 min and diluted to volume to get 5000 ppm solution, filtered through 0.45 µm filter membrane.

**Sample preparation of AN:** Similarly using optimized condition for extraction of AN, ethanol extract was obtained by refluxing 1.0 g of dried bark powder of *Acacia nilotica subsp indica* Linn. in 250 mL round bottom flask with 75 mL of 80% ethanol at 70$^{o}$C for 4 h. The contents of the flask were filtered through Whatman paper no.41 and evaporated to dryness using rotary evaporator. Dried extract yielded 0.340 g of 80% ethanol extract from reflux, which was dissolved in water and partitioned 16 times with 50 mL of ethyl acetate. All the extracts were finally pooled and evaporated to dryness using the rotary evaporator which yielded approximately 0.208 g of EtOAc extract. Accurately weighed 50 mg of ethyl acetate fractionate extract of AN was transferred into 10 mL volumetric flask containing 5 mL methanol, sonicated for 5 min and diluted to the volume to get 5000 ppm solution, filtered through 0.45 µm filter membrane.

**Chromatography:** Chromatography was performed on 100 mm x 100 mm aluminium backed TLC plates coated with 200 µm layer of silica gel $60F_{254}$ (E.Merck, Darmstadt, Germany) as the stationary phase. Standard solutions of (-)-epicatechin and sample were applied to the plates as 7.0 mm wide band, under a continuous flow of nitrogen, by means of a CAMAG Linomat V sample applicator fitted with a 100 µL syringe. The samples were applied at 8.0 mm distance from the bottom and the length of chromatogram run was 88.0 mm from the application. The mobile phase consisted of cyclohexane:ethyl acetate:formic acid in the ratio 4.0 : 6.0 : 1.0 (v/v/v). Linear ascending development was carried out in a twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 15 min at room temperature (28 ± 2$^{o}$C). After development, the plate was dried in air and densitometric scanning was performed at 280 nm with CAMAG TLC scanner using WinCATs software. The slit dimensions were 6.00 x 0.45 mm, micro and the scanning speed was 20 mm $s^{-1}$

**Method validation**

**Linear working range:** Standard solution of (-)-epicatechin of concentrations 200.0, 400.0, 600.0, 800.0, 1200.0, 1400.0 and 1600.0 ng per band were applied as bands to the same TLC plate. The procedure was repeated three times. The densitograms were recorded, and mean (-)-epicatechin peak area (Y-axis) was plotted against the corresponding concentrations (X-axis).

**System suitability:** A system suitability test was conducted to determine whether the method gave accurate results. System suitability was determined by applying a freshly prepared standard solution of (-)-epicatechin, concentration 750 ng per band, five times to the same chromatographic plate. The plate was developed under optimized chromatographic conditions, scanned and the densitograms were recorded. The measured peak area for (-)-epicatechin and their retention factors were noted for each concentration of (-)-epicatechin, and values of the mean peak area, the standard deviation (S.D.), and the relative standard deviation (% R.S.D.) were calculated.

**Table 1.**Method validation data for HPTLC quantification of (-)-epicatechin in the dried bark powder of AC and AN

| Method parameter | Value | |
|---|---|---|
| Linear range (ng per band) | 200-1600 | |
| Correlation coefficient, r | 0.9991 | |
| Limit of detection (LOD) [ng per band] | 25 | |
| Limit of quantification (LOQ) [ng per band] | 75 | |
| Instrumental precision [RSD (%) n = 6] | 1.02 | |
| Intra-assay precision on the same day [RSD (%) n = 6] | AC-1.59 | AN-1.03 |
| Intermediate precision on 3 successive days [RSD (%) n = 6] | AC-1.68 | AN-1.06 |

**Limit of detection (LOD) and Quantification (LOQ):** The limits of detection (LOD) and quantification (LOQ) were determined as the amounts for which the signal to noise ratios were 3:1 and 10:1 respectively.

**Precision:** Instrumental precision, intra-assay precision, and intermediate precision of the method were determined. Instrumental precision was measured by replicate (n=6) application of the same (-)-epicatechin standard solution (concentration 750 ng per band). Intra-assay precision was evaluated by the analysis of six replicate applications of the sample solutions of same concentration on the same day.

**Specificity:** The specificity of the proposed method was ascertained by overlaying the UV spectra of (-)-epicatechin standard and that of the samples. The peak purity of (-)-epicatechin was assessed by comparing the spectra at three different levels, namely, peak start, peak apex, and peak end positions of the band.

There was a good correlation between the spectra's obtained at each of the three positions, therefore, indicating that there is no masking by peak of other components present in the sample.

**Accuracy:** Accuracy was assessed by the measurement of recovery by the method of standard additions. The pre-analyzed extract sample was spiked with additional 80%, 100%, and 120% of a known amount of standard (-)-epicatechin and the mixtures were reanalyzed by the proposed method. This experiment was conducted in triplicate.

## RESULTS AND DISCUSSION

The response (peak area) was a linear function of the amount applied in the range of 200-1600 ng per band (Fig.1). The correlation coefficient was 0.9991, the intercept 663.1, and the slope 4.296. The LOD and LOQ were 25 and 75 ng per band respectively. In system suitability tests, the RSDs of the (-)-epicatechin peak areas and retention factors were 1.67% and 1.14% respectively. Because these values are <2%, the method is suitable for the purpose. Results from the determination of precision, expressed as the R.S.D. (%) of (-)-epicatechin peak area, are listed in table 1. The values are all <2%, indicating that the proposed method is precise.

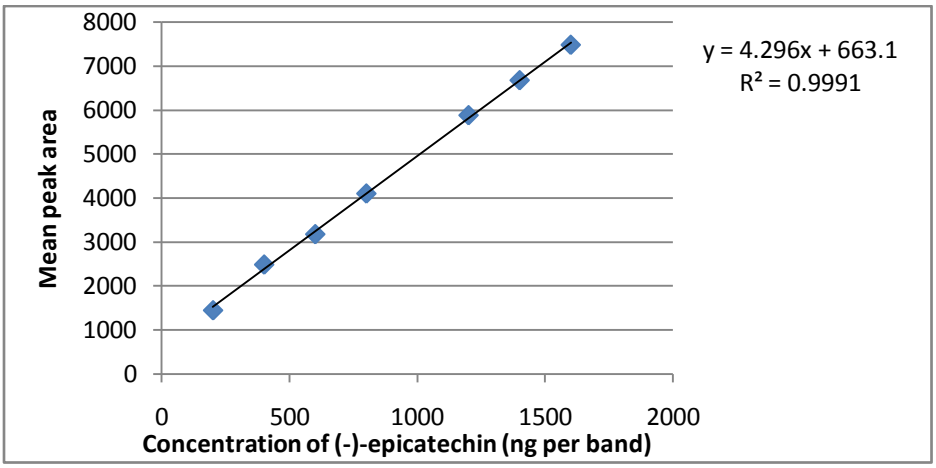



**Figure 1.** Graph of mean peak areas of (-)-epicatechin against the corresponding applied concentrations of (-)-epicatechin

A photograph of a TLC plate after chromatography of (-)-epicatechin standard and ethyl acetate fractionate of ethanol extract of the AC and AN bark powders is shown in fig. 3. Figure4 and 5 are typical densitograms of ethyl acetate fractionate of *Averrhoa carambola* Linn. bark powder, at 280nm and ethyl acetate fractionate of *Acacia nilotica subsp indica* Linn. bark powder, at 280nm respectively.

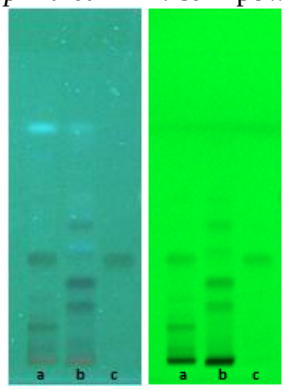



**Figure 3 .** HPTLC photo of ethyl acetate fraction of AC and AN with (-)-epicatechin standard.
(a) Ethyl acetate fractionate of AC (b) Ethyl acetate fractionate of AN (c) Standard (-)-epicatechin

Results from determination of recovery, listed in table 2 and 3, are within acceptable limits, indicating that the accuracy of the method is good.

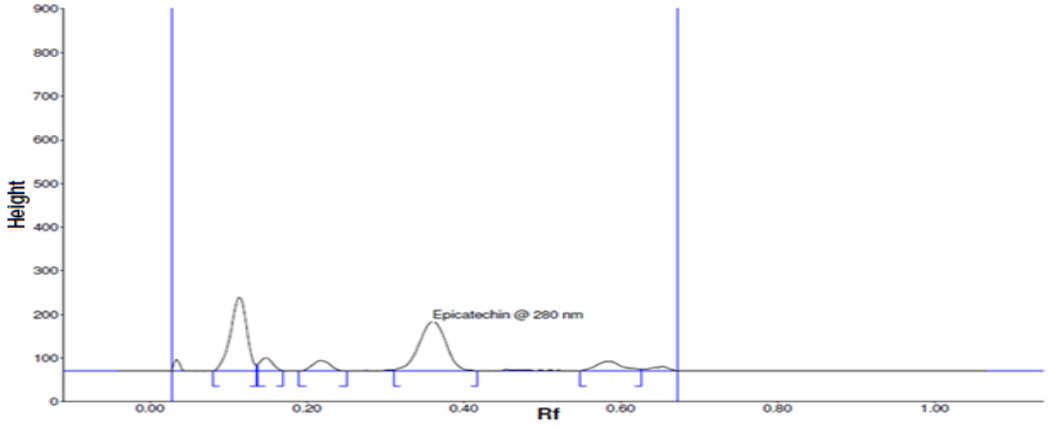



**Figure 4.** A typical densitogram of ethyl acetate fractionate of *Averrhoa carambola* Linn. bark powder, at 280nm

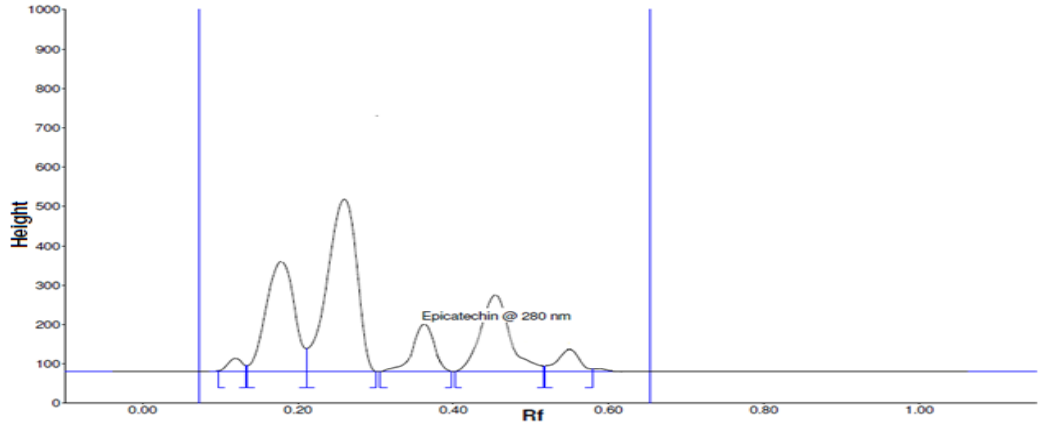



**Figure 5 .** A typical densitogram of ethyl acetate fractionate of *Acacia nilotica subsp indica* Linn. bark powder, at 280nm

**Table 2.** Results of recovery experiment for (-)-epicatechin after addition of standard (-)-epicatechin to ethyl acetate fractionate of *Averrhoa carambola* Linn. bark powder

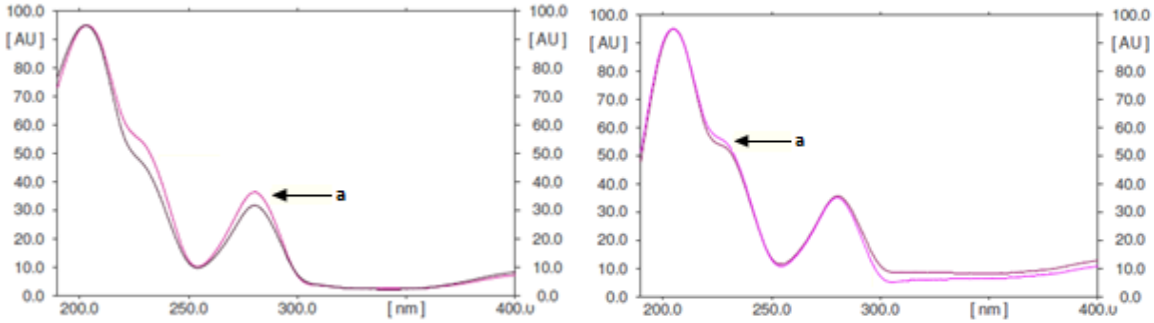
| Level | Amount (µg) of (-)-epicatechin in 2 µL of 5000 ppm sample solution (A)* | Amount of (-)-epicatechin added (B) (µg) | Amount of (-)-epicatechin present in mixture (C) (µg) | | | Mean ± S.D. (C) | % Recovery (D) |
|---|---|---|---|---|---|---|---|
| 0 | 0.744 | 0 | 0.744 | 0.739 | 0.748 | 0.744 ± 0.005 | --- |
| 1 | 0.744 | 0.32 | 1.029 | 1.040 | 1.033 | 1.034 ± 0.006 | 90.63 |
| 2 | 0.744 | 0.40 | 1.109 | 1.115 | 1.118 | 1.114 ± 0.005 | 92.50 |
| 3 | 0.744 | 0.48 | 1.174 | 1.168 | 1.182 | 1.175 ± 0.007 | 89.79 |

**Table 3.** Results of recovery experiment for (-)-epicatechin after addition of standard (-)-epicatechin to ethyl acetate fractionate of *Acacia nilotica subsp indica* Linn. bark powder

| Level | Amount (µg) of (-)-epicatechin in 6 µL of 5000 ppm sample solution (A)* | Amount of (-)-epicatechin added (B) (µg) | Amount of (-)-epicatechin present in mixture (C) (µg) | | | Mean ± S.D. (C) | % Recovery (D) |
|---|---|---|---|---|---|---|---|
| 0 | 0.717 | 0 | 0.712 | 0.720 | 0.718 | 0.717 ± 0.005 | --- |
| 1 | 0.717 | 0.32 | 0.998 | 1.007 | 1.010 | 1.005 ± 0.006 | 90.00 |
| 2 | 0.717 | 0.40 | 1.079 | 1.092 | 1.085 | 1.085 ± 0.007 | 92.00 |
| 3 | 0.717 | 0.48 | 1.162 | 1.149 | 1.157 | 1.156 ± 0.007 | 91.46 |

*indicates mean of three readings, #D = [(C-A)/B] x 100

The (-)-epicatechin peak was well resolved from other phytochemicals present in the sample, indicating there was no interference from the other components present, that is, no other components of the sample co eluted with the (-)-epicatechin peak. Specificity was confirmed by overlaying the UV spectra obtained from a standard solution of (-)-epicatechin and those from the start, apex, and end of the (-)-epicatechin peak obtained by chromatography of EtOAc fractionate of AC and AN.




(i) (ii)

**Figure 2.** Overlay of (-)-epicatechin (a) and ethyl acetate fraction of (i) AC and (ii) AN

The use of cyclohexane :ethylacetate: formic acid (4.0 :6.0 :1.0), gives good resolution of (-)-epicatechin with $R_f$ 0.36. A solution of (-)-epicatechin standard had an absorbance maximum at 280 nm so detection was carried out using UV light at this wavelength. Validation of the method showed that linearity, precision, and accuracy were satisfactory. Recovery of (-)-epicatechin at three levels was 90.97% for AC and 91.15% for AN, indicating that the method is accurate, the method is specific for (-)-epicatechin because it resolved well in the presence of other phytochemicals in AC and AN.

## APPLICATIONS

Literature survey reveals work carried out on separation, purification of phytochemicals from leaves and fruit of these plants. However no HPTLC method has been reported in literature for quantitation of (-)-epicatechin from *Averrhoa carambola* Linn. and *Acacia nilotica subsp indica* Linn. bark powders. A single method is developed to determine (-)-epicatechin simultaneously in *Averrhoa carambola* L. and *Acacia nilotica subsp indica* L., which is an economic as well as time saving method. The results of the study can serve as a valuable source of information and provide suitable standards for identification of these plant materials in future investigations and applications. Information from the finger print will also be helpful to differentiate AC and AN from their other closely related species.

## CONCLUSIONS

The method used in this article is accurate and sensitive and can be used as a routine quality control method for quantification of (-)-epicatechin in flavonoid enriched fraction of AC and AN.

## ACKNOWLEDGMENTS

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