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Separation and Quantification of Tannic Acid in *Bryophyllum pinnatum* (Lam.) Kurz. by High Performance Thin Layer Chromatography

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A sensitive, selective and robust quantitative densitometric high-performance thin layer chromatographic method was developed and validated for the determination of tannic acid in the different parts of *Bryophyllum pinnatum* (Lam.) Kurz. Tannic acid was used as a chemical marker for the standardization of *Bryophyllum pinnatum* (Lam.) Kurz. plant extracts. The separation was performed on silica gel 60 F_{254} TLC plates using toluene-ethyl acetate-formic acid (5:4:1 v/v/v) as mobile phase. The quantitation of tannic acid was carried out using the densitometric reflection/absorption mode at 270 nm. A precise and accurate quantification can be performed in the linear working concentration range of 20-100 µg mL^{-1} with good correlation (r^2 = 0.997). The method was validated for, precision, recovery, robustness, limit of detection (LOD) and quantitation (LOQ) as per ICH guidelines. Specificity of quantitation was confirmed using retention factor (R_f), UV spectral correlation of marker compound (tannic acid) in sample track.

Key Words: *Bryophyllum pinnatum*, Tannic acid, HPTLC, Validation.

INTRODUCTION

The complex contents of plant extracts make the quantitative analysis of herbal drugs difficult. Routine qualitative analysis of plant extracts is mainly based on thin-layer chromatography (TLC)[1,2]. *Bryophyllum pinnatum* (Lam.) Kurz. (Family-Crassulaceae) is commonly known as Parnabija in Sanskrit, life plant, air plant (Mexican), love plant, canterbury bells, cathedral bells, is a perennial herb growing widely and used in folkloric medicine in tropical Africa, India, China, Australia and tropical America[3]. A number of active compounds, including phenols, flavonoids, glycosides, steroids, bufadienolides and organic acids, have been identified in *Bryophyllum pinnatum* (Lam.) Kurz. The leaves of this plant contain bryophyllin, potassium malate, ascorbic acid, malic acid and citric acid[4]. The plant is rich in both macro and micro elements, vitamins, calcium, phosphorus, ascorbic acid, inulin and other compounds like flavonoids, anthraquinones, xanthones, bryophyllin A and B[4]. In traditional medicine, the leaves of this plant have been reported to possess antimicrobial, antifungal, antiinflammatory, analgesic and antihypertensive activities. The methanol extract of the leaf of the plant has also been reported to have histamine receptor (H1) antagonism in the ileum, peripheral vasculature and bronchial muscle. Studies have shown the relative important effect of some medicinal plants on the activities of central nervous system[4]. *Bryophyllum pinnatum* contain abundance amount of phenolic compound and so far, HPTLC method has not been reported for quantitation of tannic acid (Fig. 1) from different parts of *Bryophyllum pinnatum* (Lam.) Kurz. Therefore, in the present work an HPTLC method for the quantitative analysis of tannic acid has been established and has been validated as per ICH guidelines[5,6].

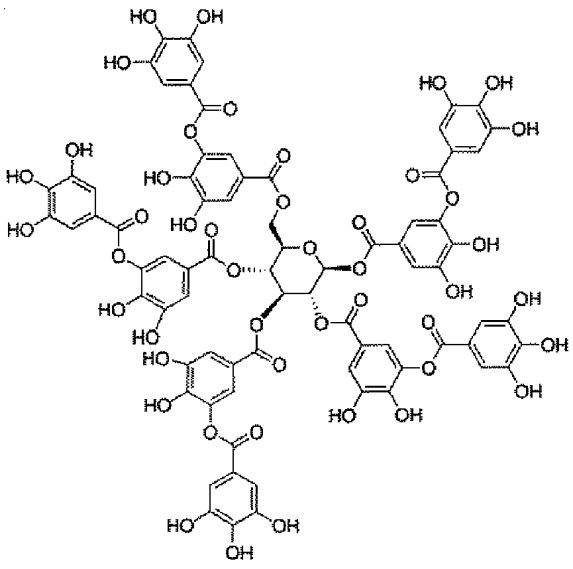


Fig. 1. Structure of tannic acid

EXPERIMENTAL

The plant material of *Bryophyllum pinnatum* (Lam.) Kurz. (*B. pinnatum*) was collected in the month of April 2011, from Kalyan, Maharashtra, India and identified by the Blatter Herbarium, St. Xavier's College, Mumbai. A voucher specimen (24919) has been deposited in the herbarium of the institute. The collected plant material was air dried under shade. Different parts of the plant were separated as root, stem and leaf and powdered to 40 mesh. Estimation of the active constituents was carried out on root, stem and leaf.

All the solvents used were of analytical grade and purchased from Hi Media (Mumbai, India). Pre-coated silica gel 60 F_{254} TLC plates (10 cm × 10 cm; 20 cm × 10 cm; E Merck, Darmstadt, Germany) were used for optimization of the analytical method. The marker compound, tannic acid (TA) (purity 99 %) was gift from Dr. Namrata Goray.

Standard stock solution and sample preparation: Standard stock solution of tannic acid was prepared by dissolving 10 mg/10 mL in methanol and filtered through 0.45 µm filters. Working stocks for calibration studies were prepared by dilution method.

Dried and finally milled root, stem and leaf (1000 mg) of *B. pinnatum* were extracted in methanol (3 mL × 5 mL, 12 h for extraction every time) and centrifuged at 5000 rpm for 10 min. The supernatants were filtered with 0.45 µm filter and used for TLC analysis.

Chromatographic conditions: Chromatography was performed on aluminum TLC plates coated with silica gel 60 F_{254} (Merck # 5554). Before use, plates were pre washed with methanol and dried in an oven at 105 ºC. Samples (10 µL) were spotted as 8 mm, starting 10 mm from edge of the plates, by means of a Camag Linomat V sample applicator. The plates were developed up to a distance of 90 mm from the base of plate in Camag twin-trough chamber previously equilibrated with mobile phase for 20 min. The mobile phase consisted of toluene-ethyl acetate-formic acid (5:4:1 v/v/v). The chromatographic conditions had been previously optimized to achieve the best resolution and peak shape. After development, plates were dried under air current at room temperature and densitometric evaluation of the plates was performed at 270 nm in reflectance-absorbance mode using deuterium lamp with a Camag Scanner 3.

The identity of the band of tannic acid in the sample was confirmed by overlaying the chromatogram of sample with that of the tannic acid and by comparing their R_f. Concentrations of the compounds chromatographed was determined from the intensity of the reflected light. Evaluation was *via* peak areas with linear regression.

Method validation: Validation of the quantitative TLC method includes the evaluation of following parameters such as linearity, limit of sensitivities, specificity, precision and accuracy and robustness according to the ICH guidelines[5,6].

Linearity and quantification: Working stock solutions were prepared by dilution to give solutions containing tannic acid in the concentrations of 20, 40, 60, 80 and 100 µg mL^{-1}. 10 µL of each standard solution was spotted on the TLC plate. Each concentration was spotted thrice on TLC plates. The calibration curves were prepared using the least-squares method, for independent variable (X) the absolute amount (µg $band^{-1}$) and dependent variable (Y) the peak area of tannic acid. Regression analyses test of the compound was performed. The curves confirm the significant linear relationship between the concentration and the peak area.

10 µL of root, stem and whole plant extract solution were taken and applied on TLC plates in triplicate with similar band pattern (as described earlier in chromatographic section). The experimental parameters were identical for all the above analysis. A calibration curve of standard as prepared above was used to calculate the per cent content of tannic acid in the sample.

Limit of detection and quantification: The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated for tannic acid using the linear regression equation. Following equations were applied: $LOD = 3S_{y,x}/b$ and $LOQ = 10S_{y,x}/b$, where $S_{y,x}$ is the standard deviation of the Y-value distribution around the regression line and b is the slope of the calibration curve.

Specificity: The specificity of the method was ascertained by co-analyzing standard and sample. The band for tannic acid in sample was confirmed by comparing the R_f and absorption spectra of the spot to that of standard. The peak purity of tannic acid peak in sample track of ethyl acetate extract was assessed by comparing the spectra at peak start, peak apex and peak end positions of the band.

Precision: Precision of the method was checked by repeated scanning (n = 7) of the same spot of tannic acid seven times each. The repeatability of sample application and measurement of peak area were expressed in terms of % RSD. Variability of the method was studied by intra-day precision and inter-day precision.

Recovery: The accuracy of quantitation was assessed in a recovery study. For this purpose, a sample with known tannic acid content was diluted, applied in triplicate onto the plate (10 µL) and individually spiked with three different amounts of tannic acid to reach a final content in the lower, middle and upper range of pseudo- linear calibration curve. Three different spiking levels (0, 50 and 100 %) of the standard stock solutions of tannic acid were prepared to calculate recovery.

Robustness: The robustness of the method was determined by introducing small changes in certain chromatographic parameters. Mobile phase having toluene-ethyl acetate-formic acid (5:4:1 v/v/v) were tried with variation of 0.5 % v/v in each solvent. Time gap between spotting to chromatography, from chromatography to scanning was varied from 0, 10, 30 min. Robustness was performed at two levels; 40 and 60 µg $band^{-1}$. At a time only one parameter was varied while the rest were kept constant. The effects on the results, *i.e.*, peak areas were examined. The standard deviation (RSD %) of peak areas was calculated for each parameter.

RESULTS AND DISCUSSION

In the present study, we quantified marker compound tannic acid in different parts of *B. pinnatum* by TLC densitometric method using silica gel HPTLC. The developed method was validated as per the ICH guidelines (Tables 1-3). Mobile phase composition was optimized by introducing small

TABLE-1
OVERVIEW OF METHOD PARAMETERS FOR THE QUANTITATION OF TANNIC ACID IN *B. pinnatum*

Parameters	Tannic acid
Retention factor (R_f)	0.44 ± 0.05
Linearity	
Working concentration range	20-100 µg mL^{-1}
Regression equation[a]	Y= 27.24x + 6.77
Correlation coefficient (r^2)	0.997
Sensitivities	
Limit of detection (LOD)	19.21 ng
Limit of quantitation (LOQ)	77.48 ng
Precision and accuracy[b]	
Instrumental (RSD %)	0.89 ± 0.07
Intra-day[b] (RSD %)	1.21 ± 0.03
Inter-day[b] (RSD %)	1.86 ± 0.08

[a]Five data point each in triplicate; x-amount of compound (µg $band^{-1}$); Y peak area in AU. [b]n = 5.

TABLE-2
RESULTS OF RECOVERY STUDY

Plant Part	Amount of tannic acid present in the sample (mg)	Amount added (mg)	Recovery (%)	Mean Recovery (%)	RSD (% n=3)
Root	0.112	–	104.05	102.60	1.55
		0.056	102.78		
		0.112	100.96		
Stem	0.040	–	97.22	102.44	4.90
		0.020	103.15		
		0.040	106.95		
Leaf	0.082	–	99.75	100.87	6.58
		0.041	100.18		
		0.082	102.69		

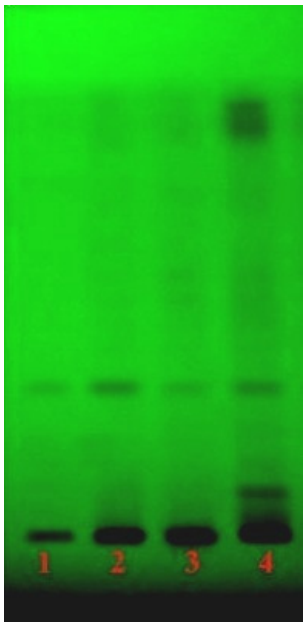
TABLE-3
ROBUSTNESS TESTING TO ACCESS THE STABILITY OF THE TLC METHOD (n = 5)

Parameters	RSD (%) of peak area of tannic acid
Mobile phase composition	1.89
Time gap between spotting and plate development	0.46

changes in the composition of toluene: ethyl acetate: formic acid. It was found that tannic acid resolved well at R_f 0.44 (Fig. 2) in the solvent system of toluene: ethyl acetate: formic acid (5:4:1) from other components of the sample extract. The identity of the band for tannic acid in the sample extract was confirmed by overlaying their UV absorption spectra with tannic acid using CAMAG TLC Scanner 3 with WINCATS software. The purity of each of these bands in the sample extract was confirmed by comparing the absorption spectra recorded at start, middle and end positions of the band.

Linearity: A good linearity was achieved in the concentration ranges of 20-100 µg/spot for tannic acid. The regression equations and correlation coefficients was [y = 27.24x + 6.77] (R^2 = 0.997) for tannic acid (Table-1).

Instrumental precision and inter-day and intra-day precision: Instrumental precision was checked by repeated scanning of the same spot of tannic acid (80 µg) seven times each. The repeatability of sample application and measurement of peak area were expressed in terms of coefficient of variance (RSD %) and found to be 0.89 ± 0.07 for tannic acid



Track 1: Standard tannic acid. Track 2: Methanol extract of root. Track 3: Methanol extract of stem. Track 4: Methanol extract of leaf

Fig. 2. Chromatogram of tannic acid with root, stem and leaf of *Bryophyllum pinnatum* (Lam.) Kurz

(Table-1). Tannic acid (40, 50 and 60 µg/spot) was spotted both at intra-day (spotting each concentration five times within 24 h) and inter-day (spotting each concentration four times during 5 days interval separated by at least 24 h) intervals to check the precision. The results were shown in Table-1. The results were expressed as % relative standard deviation (RSD %) that indicated high precision.

Recovery: The recovery was used to evaluate the accuracy of the method. The per cent recovery as well as average per cent recovery was calculated. Recovery studies at three different levels were done on *B. pinnatum* root, stem and leaf extract by accurately spiked with various concentrations of reference solutions just prior to the extraction. The average percentage recovery at three different levels for tannic acid in root, stem and leaf was found to be 102.60, 102.44 and 100.87 %, respectively. The results were shown in Table-2.

Limits of detection (LOD) and limits of quantification (LOQ): Serial dilutions of tannic acid were analyzed by TLC method. The LOD and LOQ were obtained with the signal-to-noise ratio of 3 and 10. LOD represents the lowest concentrations of tannic acid that can be detected, whereas the LOQ represents the lowest concentrations of tannic acid that can be determined with acceptable precision and accuracy. The LOD and LOQ were found to be 19.21 and 77.48 ng/spot for tannic acid. This indicated that the new method exhibited a good sensitivity for the quantification of tannic acid from *B. pinnatum*. The concentrations of tannic acid in sample solutions were within the range of linearity. The results were shown in Table-1.

Quantitative determination: All the samples were extracted, as described above and analyzed by TLC. The content of tannic acid was determined by regression equation. The results indicated that tannic acid was detected in all the samples. The chromatogram and densitograms of tannic acid along with root, stem and leaf extract of *B. pinnatum* are represented in Figs. 2 and 3. The percentage amounts of tannic

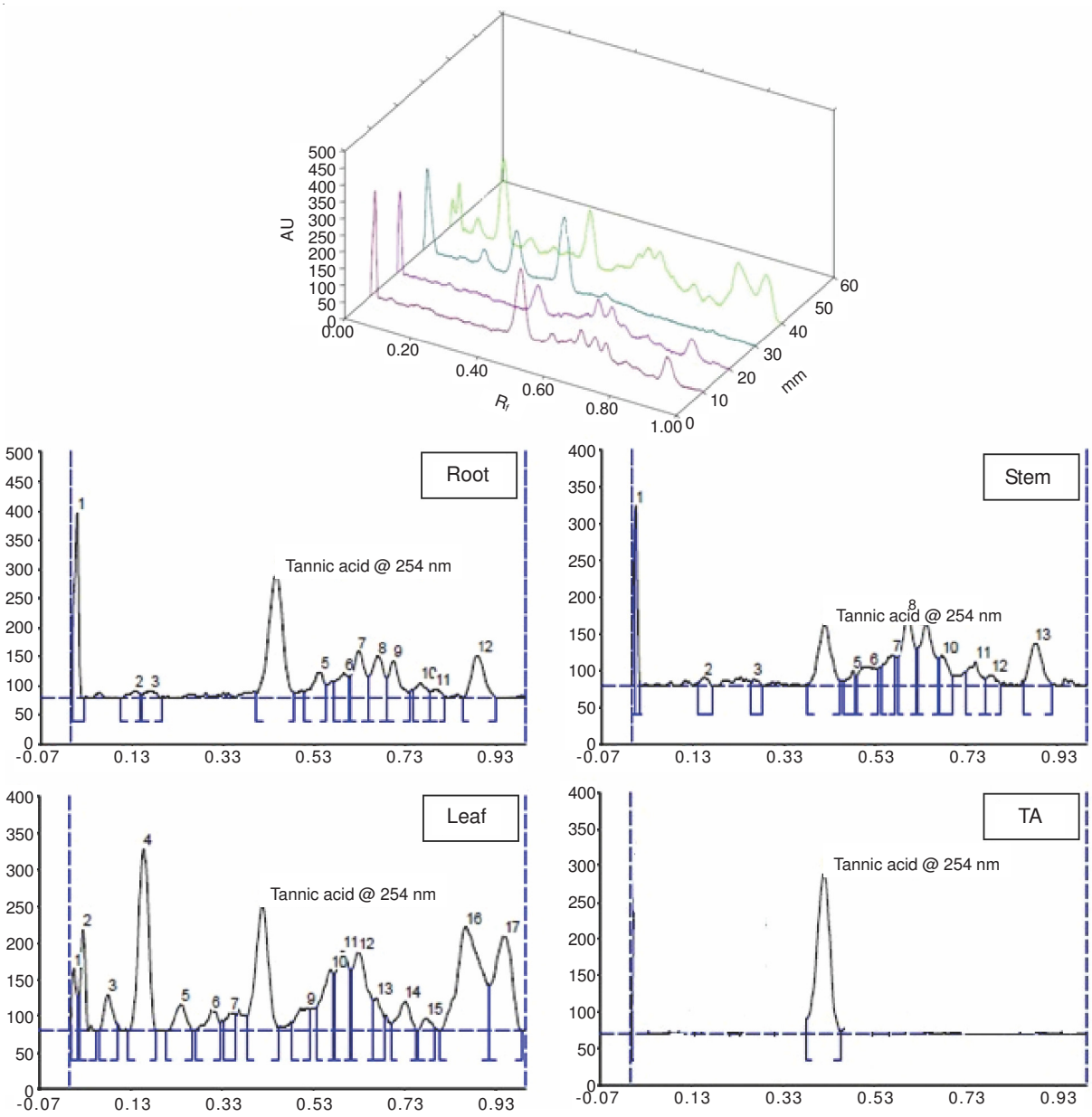


Fig. 3. Densitogram of tannic acid with root, stem, leaf and whole plant of *Bryophyllum pinnatum* (Lam.) Kurz

acid present in root, stem and leaf are presented in Table-4. Root showed maximum amount of tannic acid *i.e.*, 0.114 ± 0.09 mg g^{-1} as compare to leaf and stem, 0.082 ± 0.07 and 0.040 ± 0.08 mg g^{-1}, respectively.

TABLE-4
PER CENT CONTENT OF TANNIC ACID IN *B. pinnatum* (CALCULATED ON PLANT DRY WEIGHT BASIS)

Plant parts	Tannic acid amount quantified (mg g^{-1})
Root	0.112 ± 0.09
Stem	0.040 ± 0.08
Leaf	0.082 ± 0.07

Conclusion

We established TLC densitometric method for quantification of tannic acid from different parts of *B. pinnatum* using TLC. The method was found to be simple, precise, specific, sensitive and accurate and can be used for their quantification in the plant materials. It can be also used in routine quality control of herbal materials as well as formulations containing any or all of these compounds.

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