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Original Article

HPTLC method for the quantification of ellagic acid in different Eucalyptus species

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ABSTRACT

Ellagic acid is a naturally occurring anticancer plant product. Eucalyptus species is a rich source of ellagic acid. It possesses many important biological activities such as anticancer, hepatoprotective and DNA-gyrase inhibitor. This necessitates us to develop a suitable analytical procedure for its quantitative determination in eucalyptus species. A simple, sensitive and rapid HPTLC quantitative method of analysis has been developed and validated for ellagic acid. The quantity of ellagic acid in the curde extracts, obtained from the five common species of eucalyptus namely *E. globules Labill, E.citriodora Hook, E. eugenioides Seiber Ex Spreng, E. ficifolia F. Muell and E. pulverulenta Sims* has been determined. The collected leaves, bark and stem were extracted with hot ethanol using soxhlet extractor. The ethanolic extract was spotted on polyamide F $_{254}$ TLC plates. The plates were developed using a mixture of ethyl acetate: formic acid: water (17: 2: 3 v/v) as the mobile phase. The proposed HPTLC method was validated as per ICH guidelines for its accuracy, precision, linearity, (regression equation is y=7659x +317.4; R^2 = 0.9997), specificity and peak purity. The optimized method would be useful for both qualitative and quantitative routine analysis of ellagic acid in pharmaceutical industries, and research laboratories.

Keywords: Ellagic acid, HPTLC, Method development, Validation, Quantification.

INTRODUCTION

Ellagic acid (EA) is a phenolic compound, chemically described as 2, 3, 7, 8-Tetrahydroxy-chromeno [5,4,3-cde]chromene-5,10-dione as shown in Figure 1. EA is a naturally occurring anticancer plant product which effectively neutralizes the active form of the carcinogen, benzopyrene [(1)]. Therefore it is considered as a prototype of a new class of anticancer drug [(2-10)]. Eucalyptus species is a rich source for Ellagic acid. The leave, stem and bark of five different Eucalyptus plant species such as *E. globules Labill, E.citriodora Hook, E. eugenioides Seiber Ex Spreng, E. ficifolia F. Muell and E. pulverulenta Sims* have been collected. They all belong to the family *Myrtaceae.* Ellagic acid possesses many important biological activities such as anticancer, antioxidant activity, apoptosis inducing activity, hepatoprotective activity, antimutagenic, antihemorrhagic and DNA-gyrase inhibitors [(11-19)]. Based on these excellent biological activities, it is important to have a suitable analytical procedure for the quantitative determination of ellagic acid in *Eucalyptus* species. Recently, HPTLC method is widely used for the quantification of phytoconstituents present in various plants because of its accuracy, simplicity, cost effectiveness and rapidity [(20)]. In the present study, a new HPTLC method has been developed for the determination of ellagic acid in the ethanolic extract of leaves, stem and bark of the five species of Eucalyptus plant.

MATERIALS AND METHODS

Plant Material: Leaves, stem and bark of Eucalyptus species were collected from the Nilgiri hills, Ootcamund, Tamilnadu, India during the month of February. The plant specimen of *E. globules Labill, E.citriodora Hook, E. eugenioides Seiber Ex Spreng, E. ficifolia F. Muell and E. pulverulenta Sims* were authenticated at Botanical survey of India, TNAU Campus, Coimbatore. Tamilnadu, India.

Chemicals and Reagents: The pure ellagic Acid is purchased from Sigma Aldrich Chemicals, Bengaluru. All the reagents used in the

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development of HPTLC method were of analytical grade obtained from Merck Chemicals, India.

Instrumentation: The HPTLC system (CAMAG, Muttenz, Switzerland) consists of a CAMAG TLC scanner III integrated with Win CATS software (version 1.4.2), a Limomat 5 (fitted with 100 mL syringe), a TLC applicator connected to a nitrogen cylinder, a twin-trough glass chamber (20 X10 cm), a plate heater and a derivatization chamber. Sartorius analytical micro balance, ultra sonicator (DC1500H MRC), micropipettes and micro-pore filtration set etc were also used.

Extraction of plant material for analysis: Sun shadow dried plant parts of the leaves, stems and barks of five species of the plant were collected, shade dried and extracted (50g) with hot ethanol by soxhlet extraction process. The extraction value (% w/w) was calculated as the ratio of the weight of the concentrated extract to the weight of the dried plant part.

Preparation of sample and standard solutions: The sample solution was prepared by dissolving 25 mg of crude ethanol extract in 25 mL of ethanol. Standard solution of ellagic acid was prepared by dissolving 5 mg in 1mL of DMSO and make up to 25 mL with ethanol.

HPTLC Chromatographic Conditions:
Chromatography was performed on a 20 x 10 cm with 0.2 mm thickness pre-coated HPTLC Polyamide F_{234} glass plate. Sample and standard spots were applied to the plate as 6 mm wide band with an automatic TLC Applicator Linomat V with nitrogen flow in the dosage speed of 90 nL/s, 10 mm from the bottom. Densitometry scanner was performed on CAMAG Scanner III at 394 nm. The plates were prewashed by methanol and activated at 60 ºC for 10 min prior to chromatography. The slit dimension was kept at 5x0.45 and 40 mm sec^{-1} scanning speed was employed. The mobile phase consisted of ethyl acetate: formic acid: water in the ratio of 17:2:3 and 10 mL of mobile phase per chromatography was used. Linear ascending development was carried out in 10 x 20 cm twin glass chamber saturated with the mobile phase and then it was dried completely. Further it was derivatised with freshly prepared natural product reagent + PEG (poly ethylene glycol) solution. The chromatographic condition was given in the Table 1 and Chromatogram was shown in Figure 2.

Method Validation
The developed method was validated as per ICH guidelines, 1995 [(21)]. The validation parameters are linearity, accuracy, precision, detection limit, Quantitation limit and robustness.

Specificity: The Specificity of the method was determined by Comparing R_f Values and UV Spectra of peaks of ellagic acid in the sample and in Standard Chromatogram. The peak purity of ellagic acid was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot in overlap spectra as shown in Figure 3.

Linearity: Linearity was demonstrated from five different concentration levels of ellagic acid which was found to be linear in the range of 0.2 µg to 1.0 µg. The value was given in the Table 2. The calibration curves were shown in the Figure 4.

Accuracy (Recovery Studies): To check the degree of accuracy of the method, the recovery studies were performed by standard addition method at 40%, 80% and 120%. A known amount of standard mixture of ellagic acid was added to pre-analyzed samples and was subjected to the proposed HPTLC Method. The result of recovery studies was given in Table 3

Precision: Intraday precision (Repeatability) and Inter day precision (Reproducibility) were evaluated by carrying out six independent sample preparations. Percentage relative standard deviation (% RSD) was calculated. The results for precision was given in Table 4

Robustness: To evaluate the robustness of the developed HPTLC, small deliberate variations in the parameters of optimized method were done. The effect of ±2 % mobile phase composition and 2 different Concentration on the R_f value and area were studied. The robustness of the method was evaluated and the results were given in Table 5.

Detection Limit (DL) and Quantitation Limit (QL)
The Detection Limit (DL) is the Lowest Concentration of analyte in the sample that can be detected. The Quantitation limit (QL) is defined as the lowest concentration of the substance that can be quantified with acceptable precision and accuracy. The Detection limit and quantitation limit was calculated as DL = 3.3x σ /slope and QL = 10.0x σ /slope, here σ is residual variance due to regression. The DL and QL Values were given in Table 6

Quantification of Ellagic acid: The external standard method is generally used for quantification analysis in TLC studies as it assures accuracy and precision in quantitative analysis. The chromatograms were developed using standard ellagic acid with different concentrations ranging from 0.2 µg to 1 µg. The spots of ethanol extract of leaves, bark and stem of Eucalyptus plant with

concentration of 25 µg spot were plotted separately on Polyamide HPTLC plate. The calibration curve was obtained by plotting standard peak area against concentration. The sample peak ellagic acid area was compared with standard ellagic acid area and the percentage of ellagic acid content was calculated. The quantification result was given in Table 7.

RESULTS AND DISCUSSION

Method development: The TLC procedure was thus optimized for both standard and sample solutions. They were allowed run in different solvent systems. The mobile phase consisting of ethyl acetate: formic acid: water (17: 2: 3 v/v) had given better resolution. The spot at R_f value of 0.26 was identified as ellagic acid with the help of chromatogram of the standard compound. The spot for ellagic acid was observed in the chromatogram of the extracts along with other components. There was no interference from other components present in the chromatogram. The specificity of the method was ascertained by analyzing standard ellagic acid and extracts. The spots for the ellagic acid in sample were confirmed by comparing the R_f value and spectra of the spot with that of sample.

Validation Method: The linear regression equation was Y = 7659x + 317.4 and coefficient of correlation (R^2) for ellagic acid was found to be 0.9997. the developed method was found to be accurate and precise; the RSD values was less than 1. The detection limit and quantification limit was found to be 0.04 µg and 0.1 µg of ellagic acid respectively. The robustness of the method was evaluated; The RSD Values was less than 2.

Quantification of Ellagic acid: The HPTLC densitogram of leaves (Figure 5), bark (Figure 6) and stem (Figure 7) and HPTLC profile (Figure 8-10) were obtained using standardized HPTLC procedure. The identity of the band of ellagic acid in leaf, bark and stem extract was confirmed by comparing R_f value of the leaves, bark and stem extracts with that of standard solutions. The chromatogram of standard ellagic acid solution ranging from 0.2 µg to 1 µg had given better results (Figure 11) and hence used for the analysis. Similarly 6 readings of standard sample solution were used for the purpose of quantification as per the ICH guidelines. The Quantification analysis of data revealed interesting results that, it is highly significant to note that leaves of *E.globulus*, stem of *E. ficifolia* and bark of *E.eugenioides* are the highest yielding species among the selected five Eucalyptus species. Among these three, bark of *E. eugenioides Seiber Ex Spreng* contains considerably a larger amount of Ellagic acid.

CONCLUSION

The developed HPTLC method was simple, precise, accurate and rapid method for the quantification of ellagic acid from the leaves, bark and stem of five Eucalyptus species. The optimized method would be useful for both qualitative and quantitative routine analysis of ellagic acid in pharmaceutical and food industries, and research laboratories.

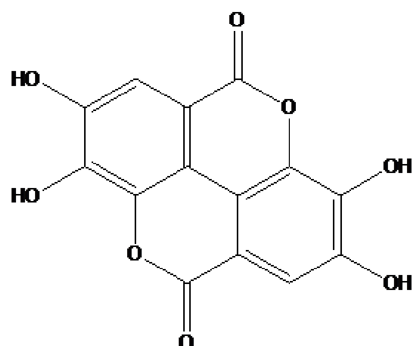

Figure 1. Structure of ellagic acid

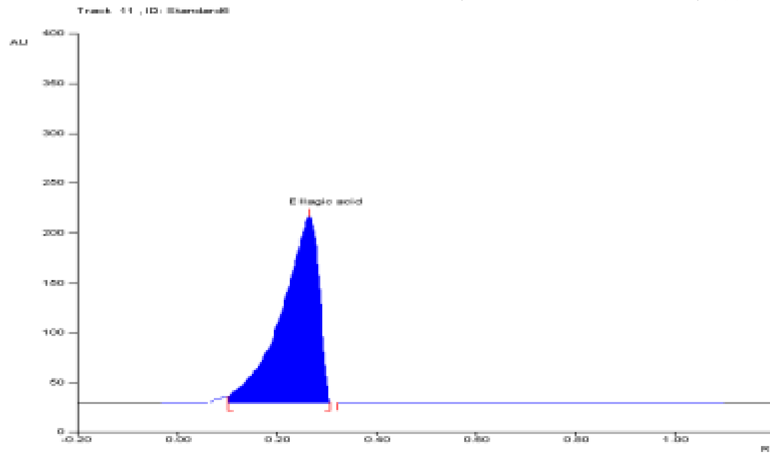

Figure 2. HPTLC Chromatogram of ellagic acid

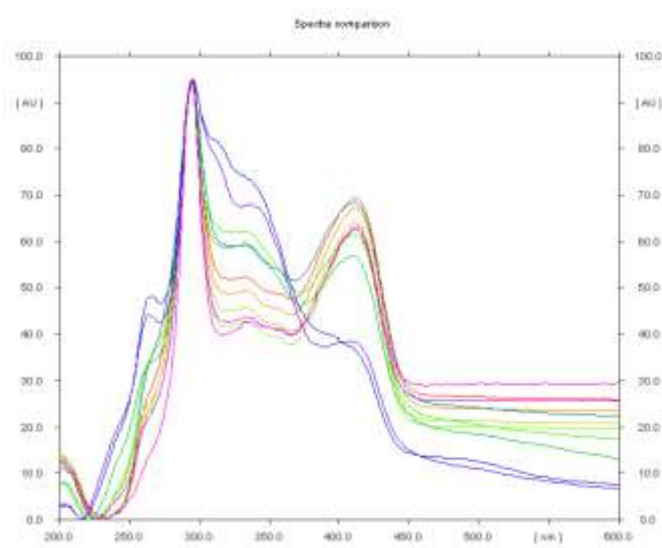

Figure 3.Overlap spectra for Ellagic acid spot

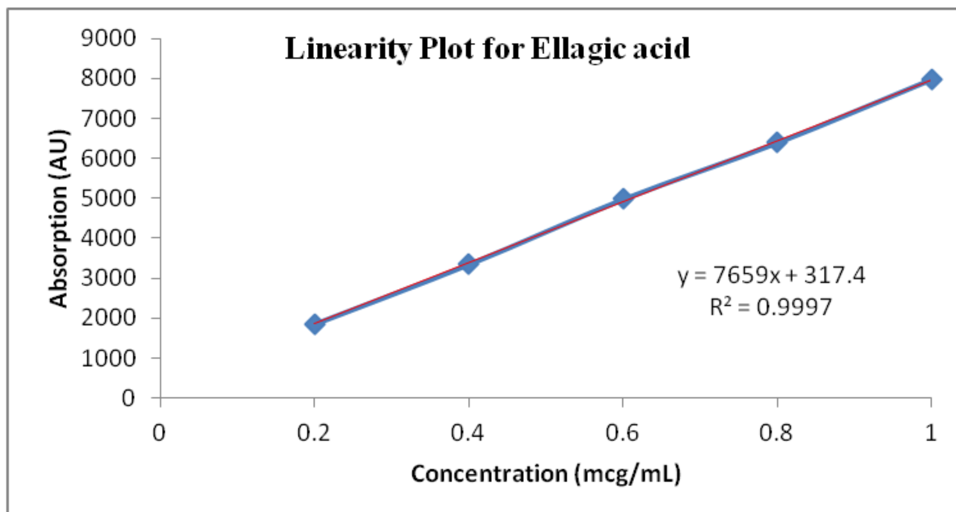

Figure 4. Linearity plot for Ellagic acid

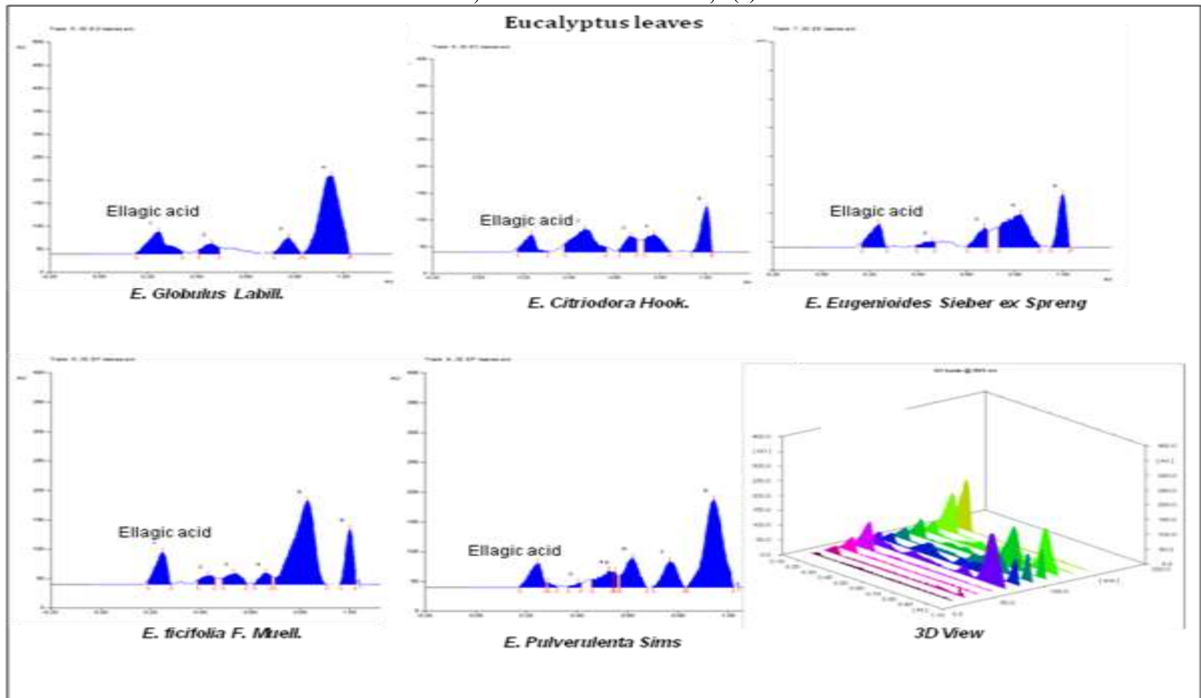

Figure 5. HPTLC Densitogram of Eucalyptus Leaves

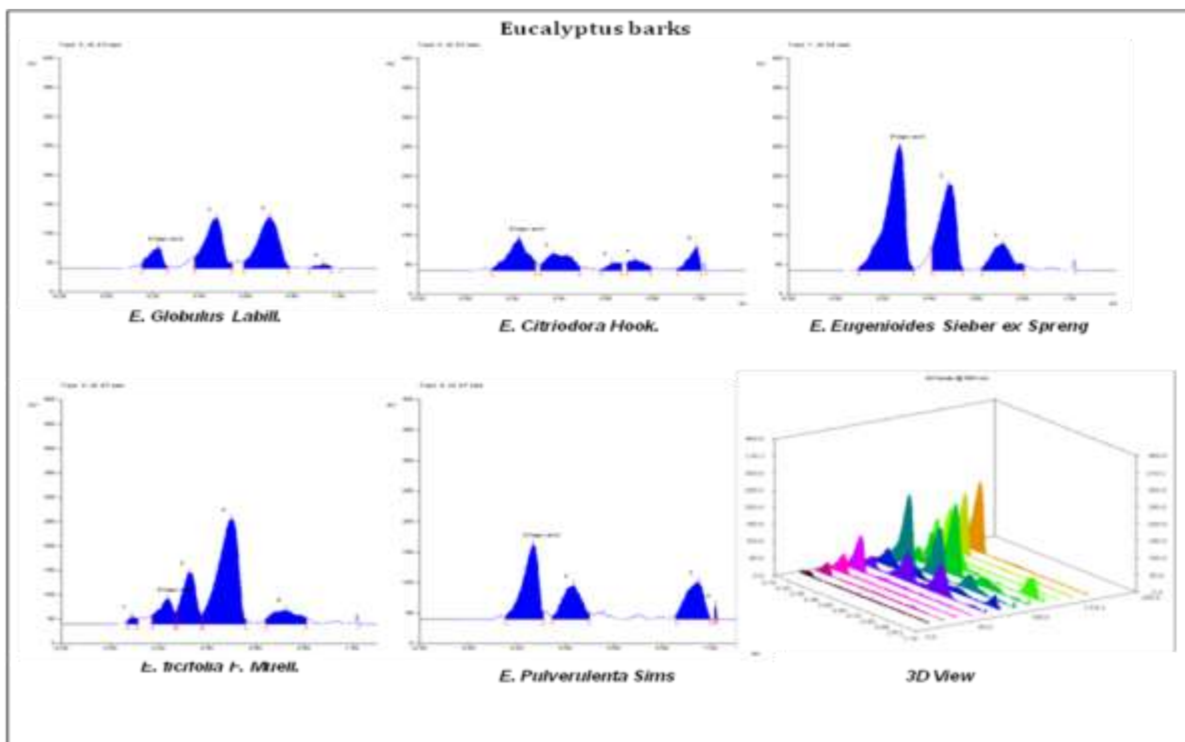

Figure 6. HPTLC Densitogram of Eucalyptus barks

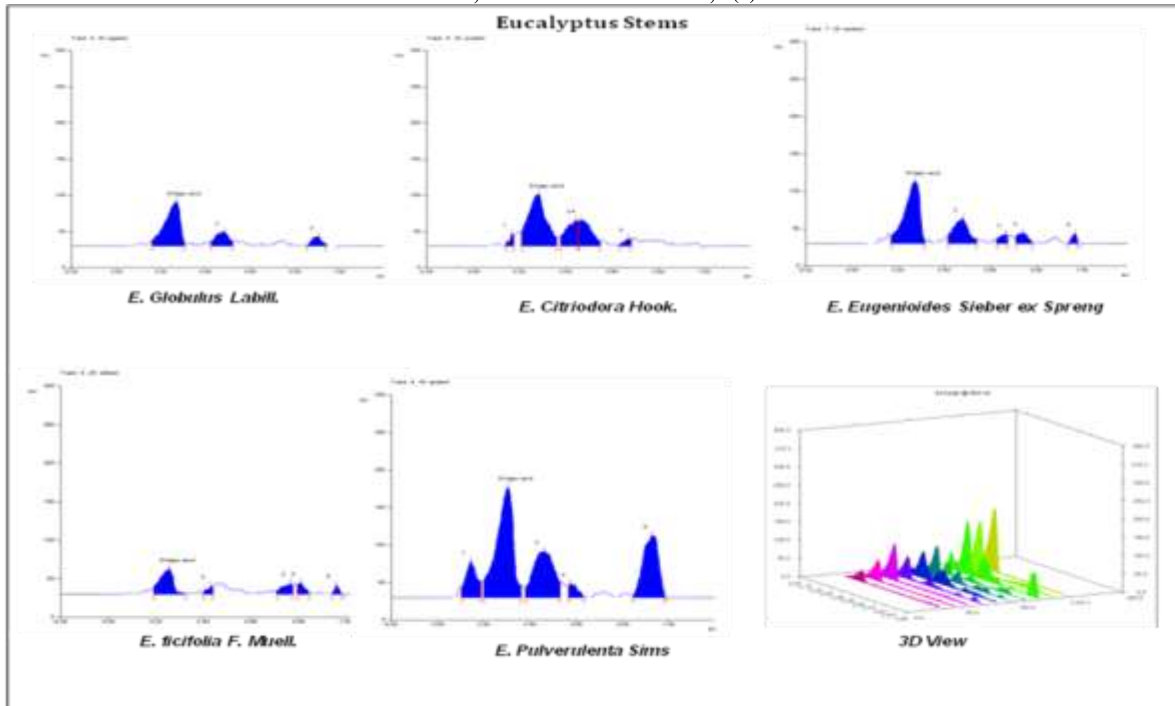

Figure 7. HPTLC Densitogram of Eucalyptus stems

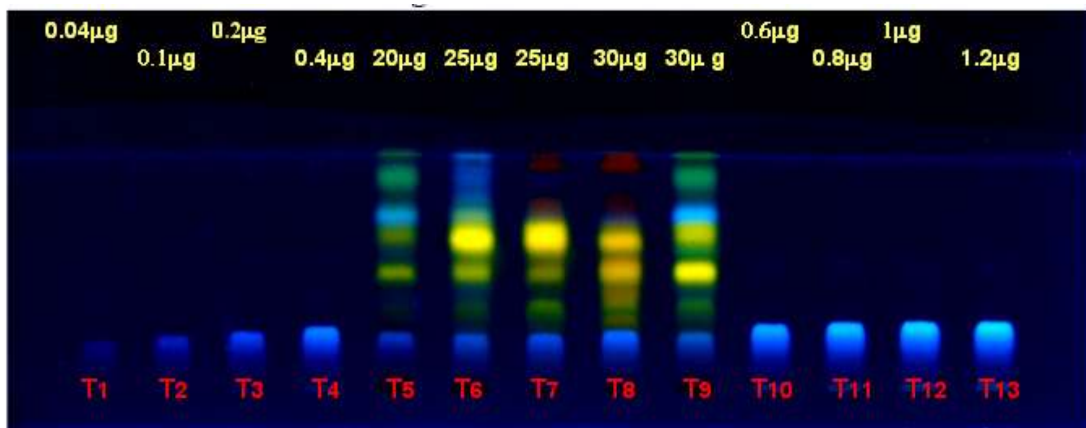

Figure 8. TLC Plate of Ellagic acid in leaves at 394nm

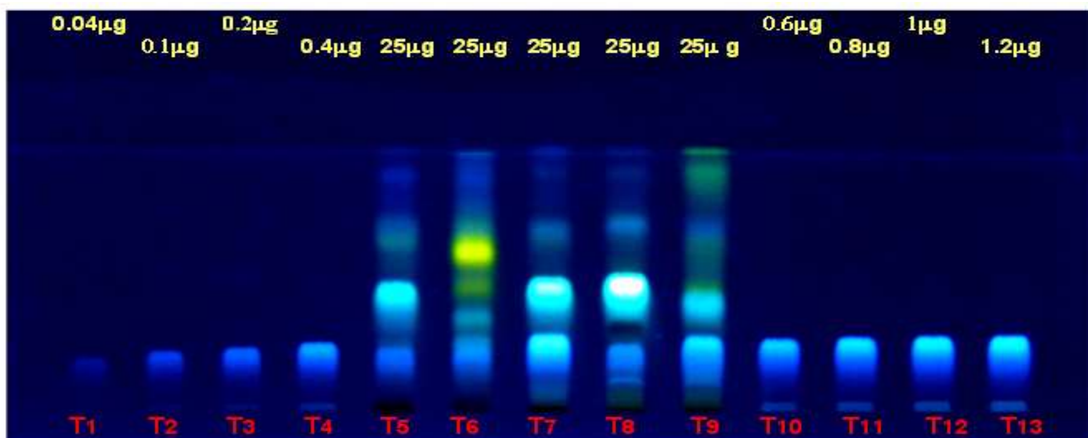

Figure 9. TLC Plate of Ellagic acid in bark at 394nm

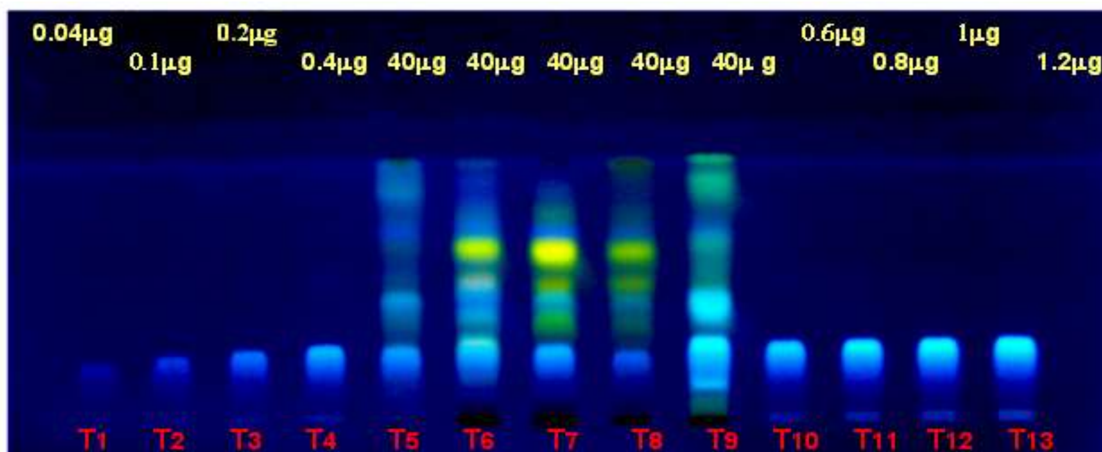

Figure 10. TLC Plate of Ellagic acid in stem at 394nm
T_1, T_2, T_3, T_4, T10, T_{11},T_{12},T_{13} -Ellagic Acid (Standard) and T_5- *Eucalyptus Globulus* , T_6 -*Eucalyptus Citriodora,* T_7-*Eucalyptus Eugeniodies,* T_8 - *Eucalyptus Ficifolia,* T_9 -*Eucalyptus Pulverulenta.* Solvent system: Ethyl acetate: Formic acid: Water (17:2:3), Detection: UV 394nm, NP reagent + PEG Solution

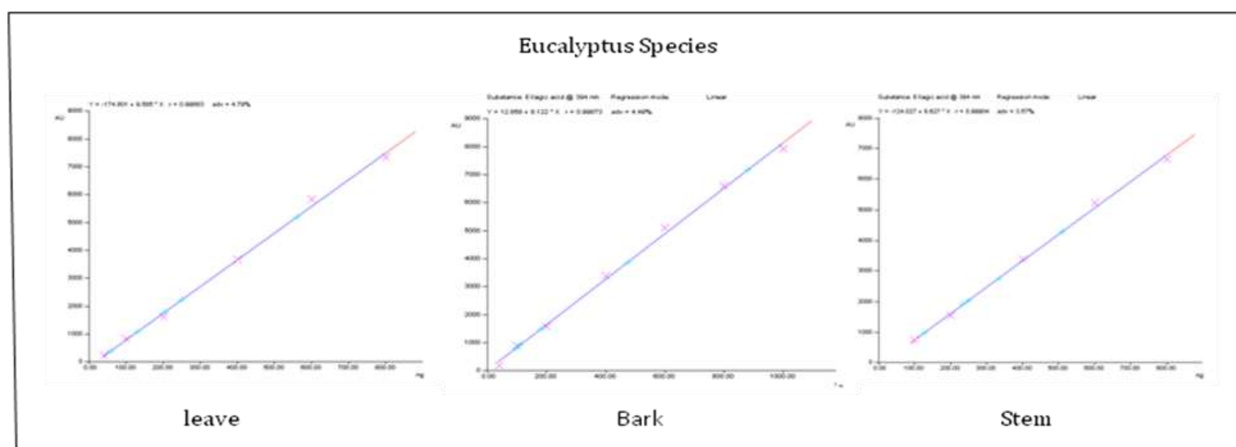

Figure 11. Standard carve of Ellagic acid in Eucalyptus leaves, bark and Stem

Table 1. Chromatographic conditions for HPTLC studies	
Parameters	**Description**
Stationary phase	Pre-coated HPTLC Polyamide F $_{234}$ glass plate.
Mobile phase	Ethyl acetate: Formic acid: Water (17:2:3
Prewashing of Plate	Methanol and activated at 60 ºC for 10 min
Development of the chamber	CAMAG Twin Trough Chamber
Chamber saturation	20 min
Sample applicator	CAMAG LINOMAT V
Dosage Speed	90 nL/s
Band Length	6 mm
Development distance	100 mm
Derivatizing reagent	Natural Product Reagent + PEG (poly ethylene glycol) solution
Drying of plate	At 110º C for 5 min
Densitometry Scanner	CAMAG TLC scanner III
Lamp	Tungsten
Wavelength	394 nm
Chromatographic Evaluation	CAMAG TLC Software Win Cats 1.4.2

Table 2. Linearity data	
Concentration (µg /mL)	**Area (AU)**
0.2	1852
0.4	3348
0.6	4980
0.8	6398
1	7986
Slope	7659
Y intercept	317.4
Correlation coefficient	0.9997

Table 3 Accuracy (Recovery Studies)		
Parameters	**Ellagic Acid (EA)**	
	% Recovery	**%RSD**
40%	99.32	0.94
80%	99.84	0.89
120%	99.26	0.91

Table 4. Precision data				
Level	**Intraday Precision (Repeatability, Accuracy) (n=6)**		**Inter day Precision (Reproducibility) (n=6)**	
	Area	**% RSD**	**Area**	**%RSD**
40	3358	0.98	3392	0.91
80	6354	0.71	6384	0.95
120	9752	0.86	9802	0.87

Table 5. Robustness for Ellagic acid (n=3)		
Ellagic acid	**Mobile phase composition**	
Amount (µg/spot)	**Ethyl acetate: Formic acid: Water (17: 2: 2.5 v/v) % RSD (n=3)**	**Ethyl acetate: Formic acid: Water (17: 2: 3.5 v/v), % RSD**
20	1.12	0.92
40	0.96	1.01

Robustness of method (n=3)

Table 6 Detection Limit and Quantitation Limit	
Sensitivity	**Ellagic Acid (EA)**
DL	0.04 µg
QL	0.1 µg

Table 7. HPTLC Quantitative Analysis of Ellagic Acid in Eucalyptus Species			
Species	**Ellagic acid content (%)**		
	Leaves	**Barks**	**Stems**
E. Globulus	**0.230**	0.031	0.050
E. Citriodora	0.034	0.191	0.040
E. Eugenioides	0.138	**0.375**	0.050
E. Ficifolia	0.080	0.027	**0.120**
E. Pulverulenta	0.220	0.124	0.090

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