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Microwave-assisted extraction of gallic acid in leaves of *Eucalyptus x hybrida* Maiden and its quantitative determination by HPTLC

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ABSTRACT

High yield and fast extraction performance ability with less solvent consumption and protection offered to thermo labile constituents are some of the attractive features of this new promising microwave-assisted extraction (MAE) technique. Therefore, MAE of gallic acid in the leaves of Eucalyptus x hybrida Maiden has been carried out and precise HPTLC method was developed for quantitative analysis of gallic acid in the leaf extracts. A range of non-polar to polar solvents were used for the MAE. A mixture of methanol: water (60:40, v/v; 20 mL) as solvent and microwave power (900 W), irradiation time of (120 s) were found to be most favorable for maximum extraction of gallic acid from the leaves. HPTLC experiments were performed on aluminum sheets pre-coated with silica gel 60 F_{254}. For achieving good separation, mobile phase consisting of chloroform: ethyl acetate: formic acid (50:50:3, v/v/v) was used. The densitometric estimation of gallic acid was carried out at λ 288 nm in remission/absorption mode. Method was validated in terms of linearity, specificity, precision, accuracy and recovery. Maximum yield 27.3% of extract was found with methanol: water (60:40, v/v) mixture, while lowest yield 2.36% was found with n- hexane when used as solvent. 100 g leaves of E. hybrida contained 114.26 µg of gallic acid on dry weight basis, when extracted with mixture (methanol: water). The proposed method would be useful for rapid quantitative determination of gallic acid in E. hybrida and its related preparations for quality assessment.

Keywords: Microwave-assisted extraction, *Eucalyptus x hybrida* Maiden, Gallic Acid, HPTLC.

INTRODUCTION

Eucalyptus is a tall evergreen tree native to Australia and Tasmania, successfully introduced worldwide, now extensively cultivated in Australia, China, India, Portugal, Spain, Egypt,

Algeria, the southern United States, and South America. Though native to Australia, its therapeutic uses have been introduced and integrated into traditional medicine systems, including Chinese, Indian Ayurvedic, and Greco-European [1].

Eucalyptus x hybrida Maiden (Family Myrtaceae), also known as Mysore gum or mainly *E. tereticorins* is extensively grown in India under the social forestry programme due to its high biomass yield in short span [2]. Chemical constituents of *E. tereticornis* includes essential oil (1, 8 –cineole or eucalyptol [1], camphene, carvone, citral, cironellal, geranyl acetate, limonene, linalool oxide) [3], phloroglucinol monoterpene derivatives (Euglobal-T1, Euglobal IIc) [3], triterpenes (ursolic acid derivatives [4]), triterpene esters (Tereticornate A and B) [5], and phenolics (caffeic, ferulic, gallic, gentisic, protocatechuic, *p*-hydroxybenzoic, p-coumaric, chlorogenic acids and *p*-hydroxybenzaldehyde, hydroquinone and vanillin) [6].
The plant has been reported to possess biological activities like anti-hyperglycemic [7], hepatoprotective [8], myorelaxant [9], antimicrobial against *S. aureus*, *S. mutans*, *E. coli* and *C. albicans* [10]. The application of medicinal plants in traditional medicine is well established and acknowledged [11]. Because of various advantages of herbal remedies, the need to search for plants of medicinal value is increasing continuously [12].

Phyto-chemical evaluation is one of the tools for the quality assessment of plants, which includes preliminary phyto-chemical screening, chemo profiling and marker compound analysis using modern analytical techniques. In the last two decades high performance thin layer chromatography (HPTLC) method has emerged as an important tool for the qualitative and quantitative phyto-chemical analysis of herbal drugs and formulations [13]. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase [13]. This also includes TLC fingerprint profiles and estimation of chemical markers and biomarkers [14].

Microwave assisted extraction (MAE) is used to accelerate the extraction process of target compounds from a variety of sources. It can be used for the extraction of compounds from various plant and animal tissues [15]. The MAE extraction method is now widely used because it is simple and rapid, involves use of lesser amount of solvent for extraction and with better yield [16].

The present study describes the extraction of gallic acid in *E. hybrida* leaves by microwave-assisted extraction method using a range of non-polar to polar solvents followed by its quantitative determination by HPTLC.

MATERIALS AND METHODS

Plant Material and Chemicals

Fresh leaves of *Eucalyptus x hybrida* Maiden were collected from Delhi region and send to NISCAIR, New Delhi for identification, specimen voucher was procured. After authentication the leaves were cleaned and dried under a gentle stream of air in the laboratory for 4-5 days till no loss in weight (temperature 25 ± 2^0C and relative humidity 65±5%) and powdered in an electric grinder. Solvents and chemicals were used of analytical grade (E. Merck, Germany). The standard gallic acid (≥ 99%) was procured from MERCK, Germany.

MAE Extraction method **[**17,18]
Powdered leaves of *E. hybrida* (5 g each) was extracted with variety of solvents ranging from non-polar to polar i.e. n-hexane, dichloromethane, ethyl acetate, acetone, methanol, methanol-water (60:40 v/v) in a domestic microwave (900W, frequency 2450MHz). Before microwave irradiation a pre-leaching time of 5 min was given to each suspension. The various experimental conditions for optimization of extraction parameters are given in Table 1.

Table 1: Conditions for MAE of gallic acid in leaves of *E. hybrida*

S.No	Powdered leaves (g)	Solvent used for extraction	Solvent vol. (mL)	Irradiation time (s)	Microwave power Input	% yield of extract
1.	5	n- Hexane	20	120	900 W	2.355
2.	5	Dichloromethane	20	120	900 W	7.311
3.	5	Ethyl acetate	20	120	900 W	9.328
4.	5	Acetone	20	120	900 W	12.837
5.	5	Methanol	20	120	900 W	26.375
6.	5	Methanol: water (60:40,v/v)	20	120	900 W	27.298

Each extract was filtered by using Whatman filter paper no. 1 and the solvents were removed under vacuum at 50°C, separately. The concentrated extracts were re-dissolved separately in methanol HPTLC studies.

Standard preparation
Standard stock solution containing 1mg mL^{-1} of gallic acid was prepared by dissolving 10 mg of gallic acid in 10 mL of methanol. The stock solution was further diluted to attain final concentration of 25 µg mL^{-1} for HPTLC analysis.

Sample preparation
Each of the concentrated extract was re-dissolved in methanol and filtered through 0.45µm filter. The concentration of individual sample extracts used for HPTLC analysis is given in Table 2.

Table 2: Yield of gallic acid in the MAE extracts

S.No	Solvent	Yield of MAE crude extract (mg)	Sample conc. (mg mL^{-1})	Yield of GA in µg per 100 g dry leaves
1.	n- Hexane	10.7	21.4	N.D.
2.	Dichloromethane	36.3	72.6	0.78
3.	Ethyl acetate	33.3	66.6	3.44
4.	Acetone	48.4	96.8	2.67
5.	Methanol	36.9	73.8	30.07
6.	Methanol- water (60:40)	39.0	78.0	114.26

HPTLC Instrumentation
Camag Switzerland HPTLC system equipped with an automatic (TLC sample applicator) Linomat 5 fitted with 100 µL syringe (Hamilton, Switzerland), TLC scanner device 4 (for multi wavelength scanning), TLC visualizer, winCATS planar chromatography manager software version1.4.5 and twin trough glass tank (20 x10 cm) was used for the analysis.
Calibration curve of Gallic acid

2 µL, 4 µL, 6 µL, 8 µL, 10 µL, 12 µL and 14 µL of gallic acid solution (25 µg ml^{-1}), corresponding to different concentrations (50,100,150,200,250,300 and 350 ng $spot^{-1}$, respectively) were applied to the TLC plate for preparing seven points linear calibration curve. The linear equation, range (ng $spot^{-1}$), slope (m), intercept (C), correlation coefficient (r), standard deviation are presented in Table 3**.**

Chromatography

The sample solution and standard solution were applied on the stationary phase *i.e.* TLC plate consisting of aluminum sheets pre-coated with silica gel $60F_{254}$ using Linomat 5 applicator. The plates were developed in twin trough glass tank using a mixture of ethyl acetate: chloroform: formic acid (50:50:3, v/v/v) as mobile phase at room temperature (28 ± 2^0C). The composition of the mobile solvent was optimized to achieve good separation. Wavelength for detection of gallic acid was selected after evaluation of complete UV spectrum of gallic acid. Quantitative analysis of the chromatogram was performed in the remission /absorbance mode at λ 288 nm for gallic acid. The slit dimension was 6.00 mm X 0.30 mm, micro scanning speed 20 mm s^{-1} and data resolution 100 µm $step^{-1}$, Figure 1.

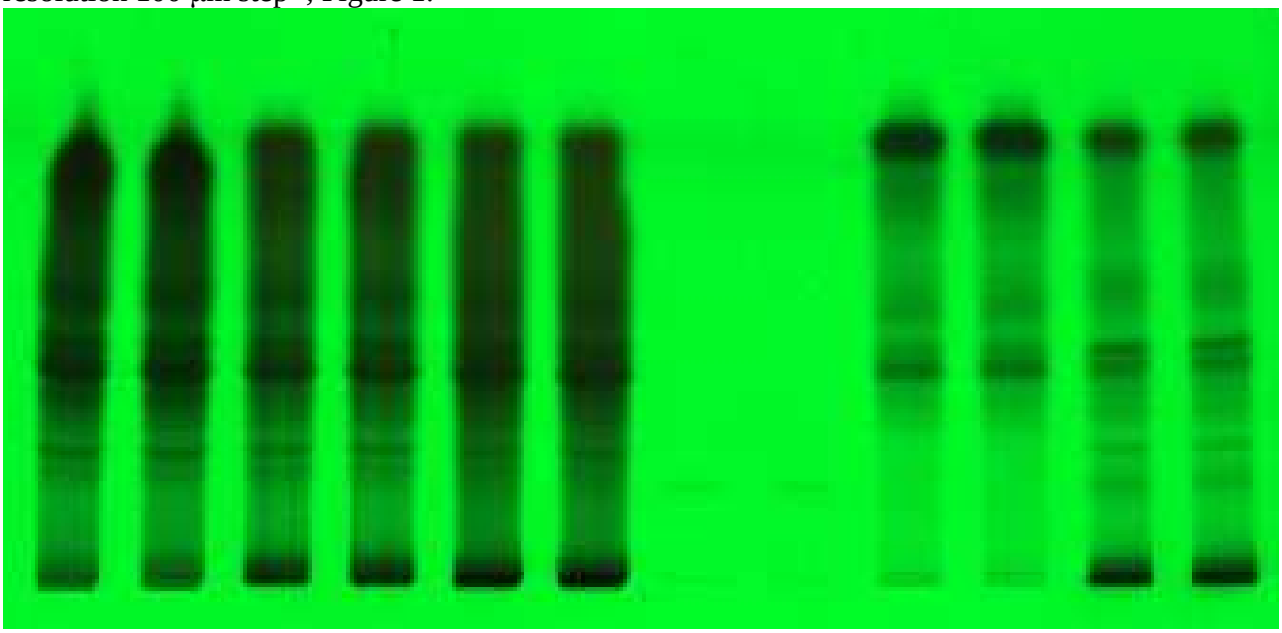

Figure 1: HPTLC fingerprint of MAE extracts and gallic acid

1=Dichloromethane extract; 2= Ethylacetate extract; 3= Acetone extract; GA= Gallic acid; 4= n-Hexane; 5= Methanol and 6= Methanol-Water(60:40) extract.

METHOD VALIDATION

In order to be a useful method for qualitative and quantitative estimation, the method was examined on the parameters of specificity, linearity, precision, accuracy and recovery. [19-22]

Specificity

The specificity of the method was determined by analyzing the sample along with the standard gallic acid. The band for gallic acid in the methanol: water (60:40) extract sample was confirmed by comparing the R_f and UV spectra of the band to that of the standard, Figure 2-4. The peak purity of gallic acid was assessed by comparing the spectra at three different levels that is peak

start, peak apex, and peak end position of the spot [22]. The data obtained is presented in Table 3.

Linearity and range

For linearity, seven different concentrations 50, 100, 150, 200, 250, 300 and 350 ng spot^{-1}of gallic acid (25 µg mL^{-1}) were applied to the HPTLC plate. A seven point calibration curve was obtained by plotting the concentration of standard gallic acid *versus* peak area Figure 5. Linear regression equations and correlation coefficient (r) values for gallic acid are given in Table 3.

Precision

Repeatability (intraday) [23]

Six replicates of 10 µL of gallic acid (25µg mL^{-1}) were analyzed by the proposed method to determine variation due to the chromatographic conditions (system precision). The % RSD of R_f and peak area were calculated and given in Table 3.

Repeatability (interday) [23]

Six replicates of 10 µL of gallic acid (25µg mL^{-1}) were analyzed in inter day (n=1, 3 & 5) by the proposed method to determine variation due chromatographic conditions (system precision). The % RSD of R_f and peak area were calculated and shown in Table 3**.**

Limit of detection and Limit of quantification (LOD and LOQ)

The gallic acid in sample extract was identified on the basis of R_f and UV- Vis spectral overlaying with the standard, Figure 4. Standard was diluted and applied on HPTLC plate to plot the calibration curve. LOD was determined based on the lowest concentration detected by the instrument from the standards while the LOQ was determined based on the lowest concentration quantified in the sample. The results are shown in Table 3.

Recovery

For percentage recovery, three known concentrations i.e. 50, 75, 100 ng spot^{-1} of standard gallic acid solution (25µg mL^{-1}) were spiked on band preloaded with 2 µL of extract (methanol:water). The bands were applied in triplicates and analyzed using the developed method. The results obtained are presented in Table 3.

RESULTS AND DISCUSSION

MAE Mechanism

Microwave-assisted extraction consists of heating the solvent in contact with the sample by means of microwave energy. The process involves disruption of hydrogen bonds, as a result of microwave-induced dipole rotation of molecules, and migration of ions, which enhance penetration of the solvents in to the matrix, allowing dissolution of the components to be extracted [24]. Usually higher dielectric constant the higher the degree of microwave absorption, it is best to choose a solvent that has a high dielectric constant as well as a high dissipation factor as evident in Table 4.

Table 4: Physical factors for commonly used solvents in MAE

Solvent	Boiling Temp (°C)	Dielectric Constant	Dissipation Factor
Hexane	68.7	1.89	0.10×10^{-4}
Dichloromethane	39.8	8.93	4117×10^{-4}
Ethyl acetate	71.1	6.02	5316×10^{-4}
Acetone	56.2	20.7	5555×10^{-4}
Methanol	64.7	32.6	6400×10^{-4}
Water	100	78.3	1570×10^{-4}

The process of microwave- assisted extraction of gallic acid in the leaves of *E. hybrida* was optimized as per reported work [13, 18]. A range of solvents like n-hexane, dichloromethane, ethyl acetate, acetone, methanol and methanol- water (60:40 v/v) were used for MAE of leaves. Solvent volume (20 mL), extraction time (120 s) and microwave irradiation power (900 W) were found to be most the suitable conditions for MAE of gallic acid in the leaves, Table 1. The maximum yield (27.3%, w/w) of extract was obtained with methanol: water (60:40, v/v) as solvent used for extraction, while lowest yield (2.36%, w/w) was found with n-hexane.

Chromatography

Different compositions of the mobile phase were tested and the desired resolution with symmetrical and reproducible peaks were achieved by using a mixture of ethyl acetate: chloroform: formic acid (5:5:0.3) as mobile phase, Figure 3**.** Identification of gallic acid in the sample extracts was done by overlaying of UV spectra and matching of R_f value with the standard gallic acid, Figure 2-4. The band of gallic acid was found to be present in all extracts Table 2 and Figure 1.

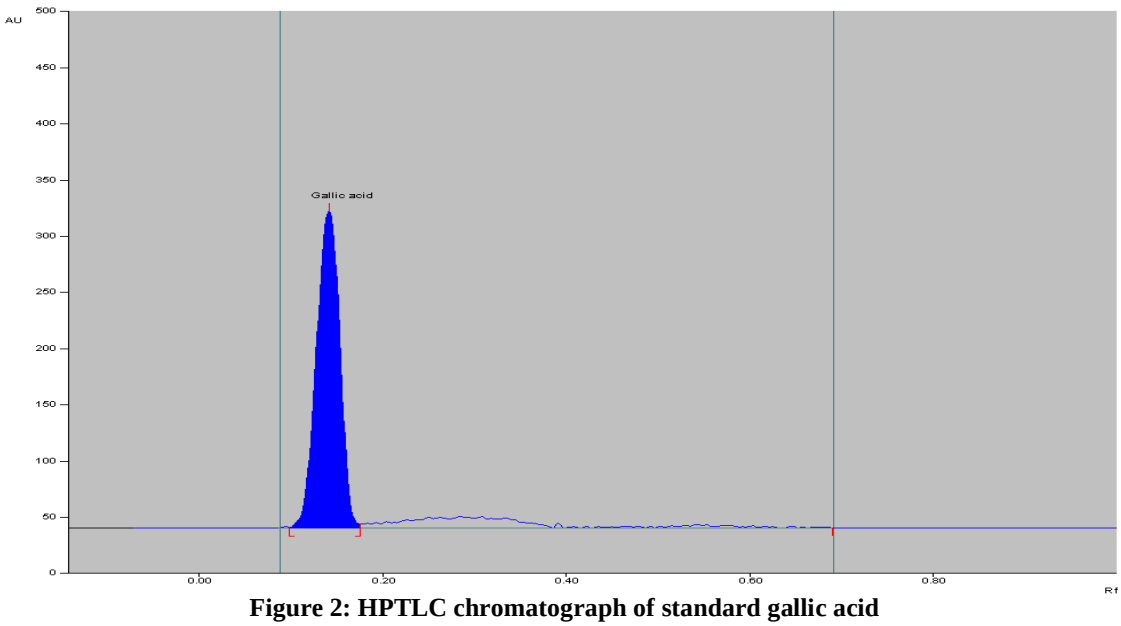

Figure 2: HPTLC chromatograph of standard gallic acid

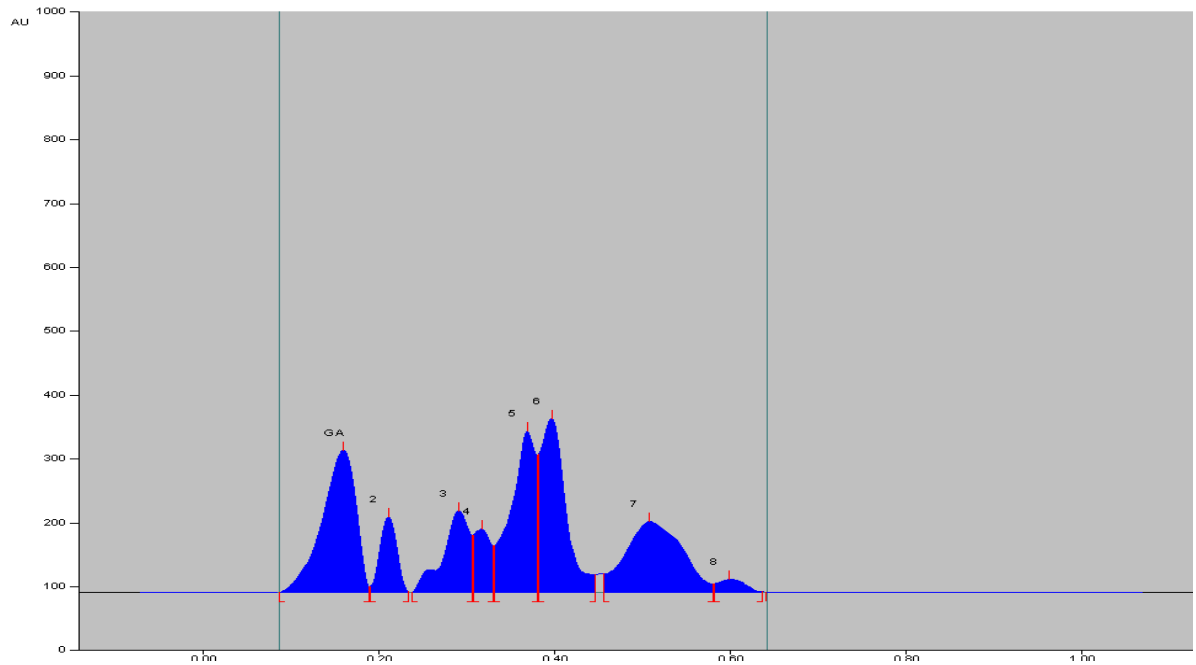

Figure 3: HPTLC chromatograph of MAE extract (methanol:water (60:40)) of *E. hybrida* leaves

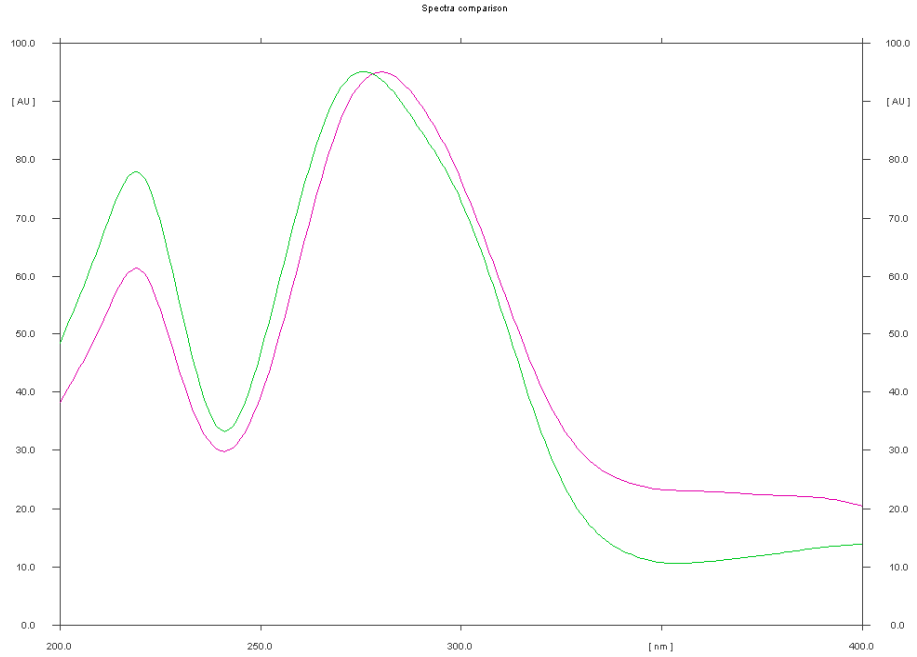

Figure 4: Overlay of UV-Vis spectra of gallic acid in standard (Red) and gallic acid in sample R_f 0.14 (Green).

The calibration plot shown in Figure 5 indicates the response is linear function of concentration *versus* peak area in the range of 50 to 350 ng $spot^{-1}$ of gallic acid. The slope, intercept and correlation coefficient were 20.06, 28.59 and 0.9989 respectively, Table 3.

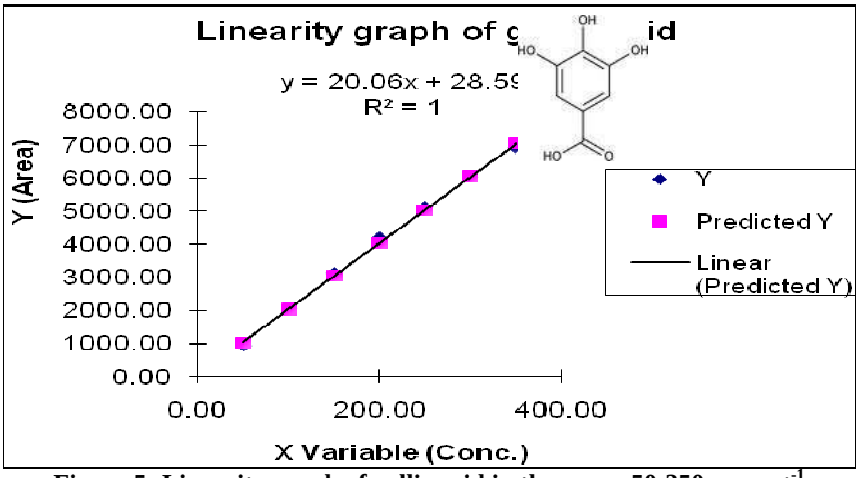

Figure 5: Linearity graph of gallic acid in the range 50-350 ng spot^{-1}

Table 3: Summary of validation parameters of gallic acid

S.No	Parameters	Results
1.	**Linearity**	
	Range (ng spot^{-1})	50 – 350
	Linear equation	y = 20.06x +28.59
	Slope (m)	20.06
	Intercept (C)	28.59
	Correlation coefficient (r)	0.9989
	r-squire (r^2)	1.0
	Standard deviation	3.04
2.	**Peak purity of eluted Gallic acid spot of sample extract**	
	Correlation coefficient, r (s, m)	0.999103
	Correlation coefficient, r (m, e)	0.997273
	Peak purity of eluted Gallic acid spot of standard	
	Correlation coefficient, r (s, m)	0.999045
	Correlation coefficient, r (m, e)	0.999012
3.	**Precision (%RSD)**	
	Intra day (n=6)	
	Repeatability of peak area	1.16
	Repeatability of R_f	0.00
	Inter day (n=6)	
	Repeatability of peak area	0.092
	Repeatability of R_f	0.00
4.	**Limit of detection(LOD)**	16.955 ng
5.	**Limit of Quantification (LOQ)**	51.380 ng
6.	**Specificity**	Specific
7.	**Recovery (%)**	90.63 - 93.37

Method validation

The method was validated for its linearity, precision, accuracy, LOD and LOQ. Good correlation coefficient (r= 0.9989) was obtained between sample and the standard of gallic acid, Table 3. The method showed acceptable precision with %RSD values less than 2% for the peak areas and R_f as evident in Table 3. Thus, the method was found suitable for the purpose of analysis. The limit of detection (LOD) and quantification (LOQ) were found 16.955 ng and 51.380 ng

respectively, which indicated the adequate sensitivity of the method, Table 3. The recovery at three different levels of gallic acid was done and the results were in the range 90.63% to 93.37% as shown in Table 3, indicated the adequate sensitivity and accuracy of the method.

Quantitative determination of gallic acid

Gallic acid was quantitatively determined in extracts of *E. hybrida* leaves using the proposed and validated HPTLC method. The amount of gallic acid in the MAE extracts (n-hexane, dichloromethane, ethyl acetate, acetone, methanol and methanol- water (60:40, v/v)) was found to be in the range of 0.7811 - 114.26 µg per 100 g dry leave of *E. hybrida* as evident in Table 2. Among all the MAE extracts, the concentration of gallic acid was found to be the highest in methanol:water (60:40, v/v) extract followed by remaining MAE extract in the order methanol> ethyl acetate > acetone > dichloromethane. Gallic acid was not detected in n-hexane extract due to the less polar nature of the extracting solvent. Further, quantification and identification of the other secondary metabolites present in leaves of *E. hybrida* by the developed method or other new method is currently under progress.

CONCLUSION

Microwave assisted extraction of gallic acid in the leaves of *E. hybrida* Maiden and its quantification by HPTLC was reported. MAE extract obtained from methanol:water mixture showed highest yield of extract as well as highest amount of gallic acid. In the proposed study 20 mL of methanol: water (60:40, v/v) mixture, microwave irradiation time of 120 s and microwave energy input of 900 W were found to be most favorable conditions for the maximum extraction of gallic acid from the leaves of *E. hybrida*. The proposed extraction technique was found to be rapid, simple, eco-friendly and economical because of its various attributes like minimization of solvents consumed, energy required and time utilized for extraction. The analytical method developed was found to be simple, sensitive and accurate and can be used for fingerprint analysis of *E. hybrida* and its preparations for quality control.

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