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Quantification of phenolic compounds in *Leucas mollissima* Wall. ex Benth through HPTLC and validation of its antioxidant potential

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The present study deals with quantification of phenolic compounds by a simple, rapid, sensitive and selective high-performance thin-layer chromatographic (HPTLC) method and evaluation of its traditional claims. A chromatographic separation was performed by using a combination of toluene: ethyl acetate: formic acid (70:30:10, v/v/v) as a mobile phase. A densitometric absorption mode has been used for the estimation of catechin and vanillic acid by comparing the peak area against the standard at wavelength 220 nm. The amount of catechin and vanillic acid was found to be 0.15% and 0.45% dry weight basis. The developed method was validated and found to be specific, linear, precise and accurate as per the International Conference on Harmonization guidelines. Antioxidant potential was evaluated by five different models having variable mechanisms of action viz., total phenolic and flavonoid content, reducing power assay, DPPH assay, deoxyribose assay and total antioxidant potential. *In vitro* potential data reveals that the species has significant potential and may be used as an alternative plant in future for the treatment of diabetic condition. However, identified bioactive compounds (catechin and vanillic acid) may be used as a reference tool for proper recognition and confirmation of right plant material and monitoring of batch-to-batch consistency of finished herbal products using *Leucas mollissima* as an ingredient.

Keywords: Catechin, HPTLC, *Leucas mollissima*, Quantification, Vanillic acid, Validation
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Leucas mollissima Wall. ex Benth (Lamiaceae) is traditionally known as "*Upanya*" and is widely distributed all over India on wastelands and roadsides.[1] It is also found in Indonesia, Japan, Malaysia, Myanmar, Nepal, Sri Lanka, Thailand, Bangladesh and Vietnam. In the traditional system of medicine, the species is used to cure urinary disorder[2], skin diseases, diabetes[3,4], gastric trouble, liver diseases cough and cold[5] and headache[6]. Leaves of *L. mollissima* are also used as an insect repellent by the tribal community of Garhwal Himalaya region, Uttaranchal, India[7]. In addition, leaf juice is externally applied in case of headaches, and a decoction is used orally for diabetes mellitus and hepatitis[8]. The hot water extract of the whole plant is used orally to treat liver diseases[9]. The phyto-constituents present in the species are alkaloids, diterpenoids, glycosides, steroids, phenolic and aliphatic compounds, which are responsible for the pharmacological potential of the plant[10,11].

Phenolic compounds are a structurally diverse set of compounds responsible for the range of therapeutic properties. They occur as a complex structure associated with the oxygenated heterocyclic ring such as benzoic acid derivatives and many others. The reactive oxygen species (ROS) generation begins with rapid uptake of oxygen, activation of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and the production of the superoxide anion radical (O_2^-)[12]. In the present study, an easy, precise and accurate HPTLC method was developed for the identification and quantification of bioactive phenolic compound, i.e., catechin and vanillic acid in *L. mollissima.* The antioxidant potential of the species was evaluated to validate the traditional claims.

Methodology

Plant material

The *L. mollissma* germplasm was collected in September from Tamiya forest, Madhya Pradesh, India (Latitude 22º20'38"N, longitude 78º40'13"E and height 938 m) (Fig. 1). Plant material was identified and authenticated by Dr Sharad Srivastava, Principal Scientist, Pharmacognosy division, CSIR-NBRI, Lucknow. Plant specimen was assigned a voucher number (LWG No. 254033) and deposited

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in the institute's repository. Arial parts were washed, shade dried and powdered (40 mesh) using an electric grinder.

Chemicals

Toluene, ethyl acetate and methanol were procured from Spectrochem Pvt. Ltd. Mumbai and HPTLC precoated silica gel 60 GF_{254} (20 x 20 cm) plates procured from Merck, India, was used. Marker compounds catechin (99%) and vanillic acid (99%) were purchased from Sigma-Aldrich, USA, for quantification studies. All other chemicals and reagents used were of analytical grade purity.

Extraction protocol for catechin and vanillic acid

The coarsely powdered aerial parts of plant sample (2 g) were extracted (cold maceration) with methanol for 24 h at room temperature (25±2ºC). Extraction was repeated thrice, filtered and pooled filtrate was dried in rotatory evaporator (Buchi, USA) under standard conditions of temperature (55±2ºC) and pressure (40 mbar) and lyophilized (Labconco, USA). The extractive yield was calculated (%) on dry weight basis.

Chromatographic conditions

Stock solution of plant sample and marker compounds of strength 10 mg/mL and 1 mg/mL, respectively, were prepared in methanol. Working solution of plant sample (1.0 mg/mL) and marker compounds (0.1 mg/mL) were freshly prepared from stock on the same day for analytical studies. Spots were applied as 6 mm-wide bands positioned 15 mm from the bottom and 15 mm from side of the plate, using Camag Linomat V automated TLC applicator with nitrogen flow providing a delivery speed of 150 nL/s from application syringe. These conditions were kept constant throughout the analysis. Following sample application, a plate was developed in a Camag twin trough glass chamber, presaturated with tertiary mobile phase up to 8 cm height. After development, the plate was dried at room temperature. Catechin and vanillic acid was quantified using Camag TLC scanner model 3 equipped with Camag win CATS IV software (slit width, 5 mm x 0.45 mm) in absorption-reflection mode[13].

Fig. 1 — A flowering twig of *Leucas mollissima*

Method validation

HPTLC method validation includes evaluation of linearity, sensitivity, precision, selectivity and robustness parameters according to the guidelines of International Conference on Harmonization (ICH) to access the performance of the method[14].

Linearity

Different dilutions were spotted in triplicate on TLC plate at the concentrations of 2, 4 and 6 µg per spot of catechin and vanillic acid. The data of peak area versus concentration was treated by linear least-square regression equation. The slope, intercept and correlation coefficient for the calibration curve were determined with 6 different concentrations. The results are expressed as percentage of the total area of identified compounds. Based on the calibration curve of catechin and vanillic acid, content was estimated in the plant sample and expressed on (%) dry weight basis.

Sensitivity

Sensitivity of the method was determined with respect to limit of detection (LOD) and limit of quantification (LOQ), calculated from the standard deviation of the response and slope of the calibration curve.

Stability

The reproducibility of method was determined by analyzing marker compound of single concentration (0.1 mg/mL) over three times in the same day. The relative standard deviation was used to evaluate the reproducibility of method within the limit of standard. The developed method was also validated for selectivity, specificity and resolution of analytes.

Precision

Interday and intraday studies were carried out to test the precision of method and expressed as relative standard deviation (%). Intraday repeatability was

tested by scanning marker compounds three times a day. Similarly, interday repeatability was assessed over three consecutive days.

Accuracy

The accuracy (marker compound addition method) of the method was determined by analyzing the percentage recoveries and mean RSD (%) of catechin and vanillic acid in the plant sample. The samples were spiked with three different concentrations: 50, 100 and 150 µg. The spiked samples were recovered in triplicate and then analyzed by the developed HPTLC method.

***In vitro* antioxidant activity**

Total phenolic[15] and flavonoid content[16] was expressed in terms of mg/g of QE (Quercetin Equivalent) and mg/g GAE (Gallic Acid Equivalent) based on the calibration curve of quercetin and gallic acid as standard. The antioxidant potential was analyzed by the DPPH radical scavenging assay[17], total antioxidant capacity[18] and ferric reducing power assay[19].

Statistical analysis

Observation for each sample was performed in triplicate. The data were recorded as mean ± standard deviation and analysis of variance (ANOVA) was used to calculate the critical f value (f-test) and the statistical significance for the analyzed catechin and vanillic acid content by Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA) software. The significance of the regression coefficient was evaluated by f-test. Differences were considered significant at $p<0.05$. Cluster analysis and correlation coefficient was estimated by PAST software, version 2.15.

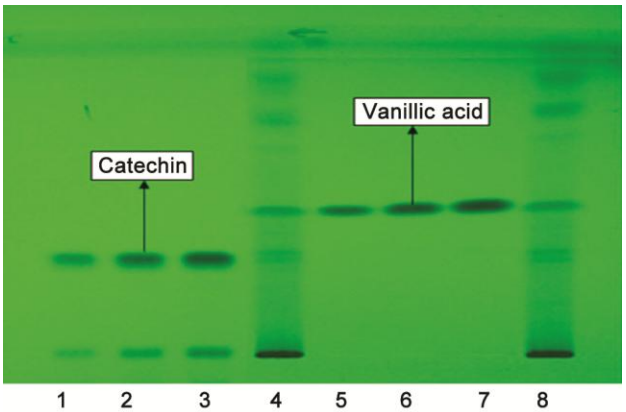

Fig. 2 — HPTLC fingerprint of *Leucas mollissima* at 254 nm Abbreviation: 1-3 Catechin, 4, 8. *Leucas mollissima*, 5-7. Vanillic acid

Results and Discussion

Quantification of catechin and vanillic acid

The HPTLC chromatogram was developed to separate the targeted marker in a tertiary solvent system of toluene: ethyl acetate: formic acid (70:30:10, v/v/v) on precoated silica gel 60 F_{254} aluminum plates, and densitometric determination was carried out at 220 nm. Catechin and vanillic acid as marker compounds were identified at R_f 0.26 ± 0.03 and 0.40±0.02 respectively (Fig. 2-4). Quantification of catechin and vanillic acid in *L. mollissima* was carried out on the basis of the calibration curve of marker compounds (Fig. 5). Six dilutions were used in concentration range of 2, 4, 6, 8, 10 and 12 µg/mL and calibration parameters were

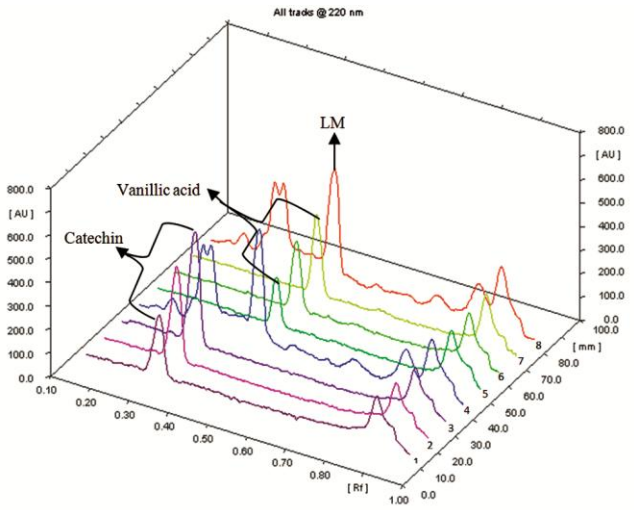

Fig. 3 — 3D Densitometric overlay spectra of *Leucas mollissima* Abbreviation: 1-3 Catechin, 4, 8. *Leucas mollissima*, 5-7. Vanillic acid

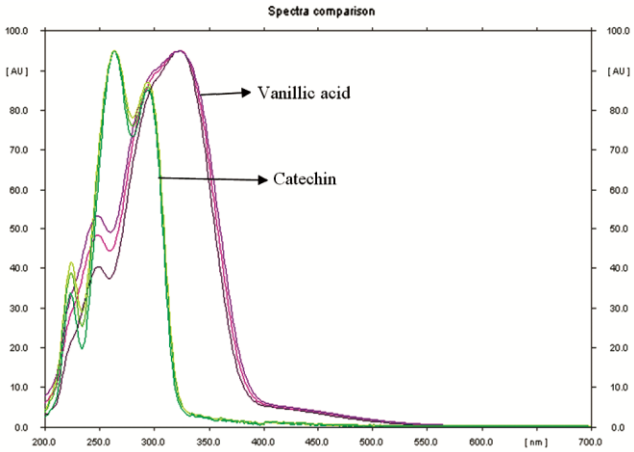

Fig. 4 — Absorption spectra of catechin (ng/mL) and vanillic acid (nf/mL)

established (Table 1). Concentration of catechin and vanillic acid was found to be 0.15% and 0.45 % per dry weight, respectively, in *L. mollissima.*

Method validation

The developed method was validated as per the ICH guidelines. The calibration curves were plotted between amounts of analytes versus average response (peak area). The correlation coefficients (r^2) for catechin and vanillic acid were 0.989 and 0.993, respectively, which indicated the high degree of correlation and good linearity of the method. LOD was considered as 3:1 and LOQ as 10:1. For catechin and vanillic acid, LOD was 6.61 and 6.64 µg $spot^{-1}$, whereas, the LOQ was found to be 20.05 and 20.12 µg $spot^{-1}$, respectively (Table 1). Precision validation of the method was analyzed by interday and intraday repeatability studies at single level using fixed concentration (0.1 mg/mL) of marker compounds. RSD (%) values (Table 2) were observed within limit, i.e., NMT 5%. Accuracy was tested through the marker compound addition method by spiking of samples at three different levels of 50%, 100% and 150%. Recovery of analytes (Table 3) shows the variation from 100.02% to 100.21%, which are in the acceptance limit of 95%-105% and hence, the method was found to be accurate and precise. The validation results signify that the developed method is suitable for the quantitative analysis of catechin and vanillic acid in *L. mollissima*.

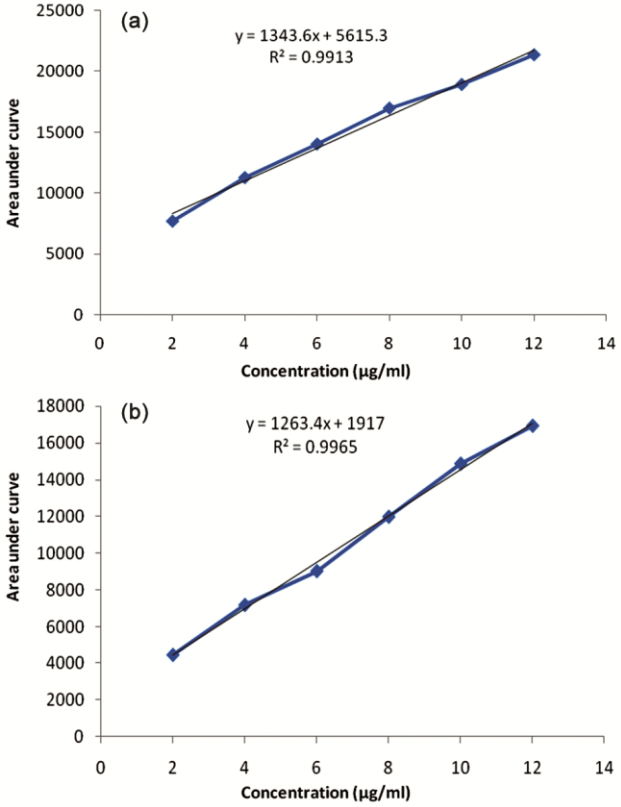

Fig. 5 — Calibration graph of catechin (a) and vanillic acid (b)

***In vitro* antioxidant activity**

Antioxidant potential of *L. mollissima* was calculated by five different models having variable mechanisms of action. viz., total phenolic and flavonoid content, reducing power assay, DPPH assay, deoxyribose assay and total antioxidant potential.

The flavonoids and phenolic content exhibit strong antioxidant potential, which depends on the formation of the complex (ferricyanide is converted to ferrous form) with metal atoms, i.e., iron and copper. Total

Table 1 — Statistical parameter for calibration catechin and vanillic acid

Parameters	Catechin	Vanillic acid
Linearity range (µg/mL)	2-12	2-12
R_f	0.26	0.40
Regression eq.	y = 1343.6x + 5615.3	y = 1263.4x + 1917
R^2	0.9913	0.9965
Slop	1343.64	1263.44
Intercept	5615.25	1917.007
Average	10983.66	10761.12
Standard deviation	5049.39	4735.71
Standard error	525.88	314.15
LOD (µg/spot)	12.41	12.36
LOQ (µg/spot)	37.41	37.47

Table 2 — Precision studies for catechin and vanillic acid

Concentration amount of standard (µg $spot^{-1}$)	Catechin				Vanillic acid			
	Intraday		Interday		Intraday		Interday	
	SD	%RSD	SD	%RSD	SD	%RSD	SD	%RSD
2	51.11	0.6697	19.311	0.281	26.34	0.587	37.38	0.92
4	42.79	0.380	39.788	0.422	47.06	0.653	33.16	0.361
6	12.12	0.086	67.039	0.548	53.02	0.585	56.88	0.603

n=3, RSD-relative standard deviations, SD-standard deviation

Table 3 — Result and statistical data for recovery studies for catechin and vanillic acid in *Leucas mollissima* RSD – relative standard deviations

Sample	Amount of Catechin present in Sample (µg)	Amount of Catechin Added (µg)	Theoretical added value (µg)	Amount of Catechin analyzed (µg)	Recovery (%)	Average Recovery	Mean RSD (%)	Amount of Vanillic acid present in Sample (µg)	Amount of Vanillic acid Added (µg)	Theoretical added value (µg)	Amount of Vanillic acid analyzed (µg)	Recovery (%)	Average Recovery	Mean RSD %)
Leucas mollissima	0.15	50	50.15	50.25	100.19			0.45	50	50.45	50.45	100.00		
	0.15	100	100.15	100.37	100.21	100.14	0.104	0.45	100	100.45	100.63	100.17	100.11	0.097
	0.15	150	150.15	150.19	100.02			0.45	150	150.45	150.72	100.17		

phenolic and total flavonoids content was determined. Results show that total phenolic (TPC) and total flavonoids content (TFC) were found to be 4.3±0.002 (mg/g) and 3.01±0.003 (mg/g) (Table 4). The ferric reducing power assay is based on the principle of increase in the absorbance of the reaction mixtures; the absorbance increases as the antioxidant activity increases[20,21]. The ferric reducing power assay of extract served as a significant indicator of its potentiality as a reducing agent, which in turns signifies its antioxidant activity[22]. Result showed that the reducing power of methanolic extract of *L. mollissima* increases linearly with increase in concentration having regression coefficient (r^2) of 0.9603 and correlation equation (y=0.0001x+0.0378). This is found similar to standards, i.e., quercetin (0.997) and rutin (0.998).

The total antioxidant assay is based on the reduction of phosphate-molybdenum (VI) to phosphate-molybdenum (V). The incubation of extracts with the molybdenum (VI) will express the presence of antioxidant components in the extract. So, this assay is very useful to predict the antioxidant activity of crude extracts on the total basis[23,24]. The total antioxidant capacity of *L. mollissima* (methanolic extract) extract was based on regression analysis of ascorbic acid, having regression equation (y=4.804x+0.136) and regression coefficient (r^2) 0.987. It is observed that the species exhibit promising *in vitro* antioxidant activity.

Identified phyto-compounds (catechin and vanillic acid) are efficient in quenching the singlet oxygen radical and the rate of quenching of singlet oxygen by catechin and vanillic acid is typically depends on the potential of species[25]. Free radical scavenging activity of DPPH is most widely used for screening of medicinal plants having antioxidant activity. The

Table 4 — Phytochemical analysis of *Leucas mollissima*

SN	Analysis	Value (mg/g)
1	Total Phenolic content {(mg/g)*GAE}	4.3±0.002
2	Total Flavonoid content {(mg/g)*QE}	3.01±0.003

n=3, ± SD

Table 5 — Analysis of antioxidant potential of *Leucas mollissima*

S/N	Plant/standard	DPPH IC_{50} (µg/mL)	Deoxy ribose assay IC_{50} (µg/mL)
1	*Leucas mollissima*	411.38 ± 0.021	25.865 ± 0.005
2	Catechin	06.04 ± 0.122	07.55 ± 0.021
3	Vanillic acid	05.241 ± 0.173	06.52 ± 0.074
4	Ascorbic acid	3.86 ± 0.057	10.37 ± 0.057

n=3, ± S

scavenging effect of DPPH radical on *L. mollissima* was concentration dependent and varied among samples as well as standards (ascorbic acid, catechin and vanillic acid). Ascorbic acid exhibits maximum inhibition of 77.57% (IC_{50} at 3.86±0.057 µg/mL), which is followed by catechin and vanillic acid having inhibition of 74.55 (IC_{50} at 6.04±0.122 µg/mL) and 72.48 (IC_{50} at 5.241±0.173 µg/mL), respectively. *L. mollissima* have IC_{50} at 411.38±0.021 µg/g (Table 5). The hydroxyl radical's potential is measured by the ability of extracts to prevent degradation of deoxyribose by the hydroxyl radicals, generated in the reaction mixture. Highly reactive hydroxyl radicals can be generated in biological systems by the Fenton reaction[26]. The deoxyribose assay of *L. mollissima* extract was measured (IC_{50} value 25.865±0.005) based on the regression analysis of ascorbic acid, having regression equation (y = 4.804x+0.136) and coefficient; r^2 = 0.987 (ascorbic acid was taken as the positive control). Results show that capacity was

observed in *L. mollissima* {IC $_{50}$ value 25.865±0.005 µg/g ASE (ascorbic acid equivalent) (Table 5)}. It is observed that species exhibit promising *in vitro* antioxidant activity potential.

Conclusions

A simple, rapid, accurate and precise HPTLC method has been developed for simultaneous quantification of catechin and vanillic acid in *L. mollissma*, hence, it can be recommended for the routine analysis of the herbal drugs. In conclusion, it is evident from the current study that *L. mollissma* contains considerable amount of catechin and vanillic acid, which in turn reflects its rich antioxidant activity. Further, the identified marker compound can be used to evaluate the batch-to-batch consistency of the herbal products made out of this plant.

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Conflict of interest

The authors do not have any conflict of interest.

Authors' contributions

PKS-Manuscript writing, HPTLC analysis and data interpretation; AM-Manuscript correction and writing; AS-In vitro analysis and manuscript writing; SS-Research design and data authentication

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