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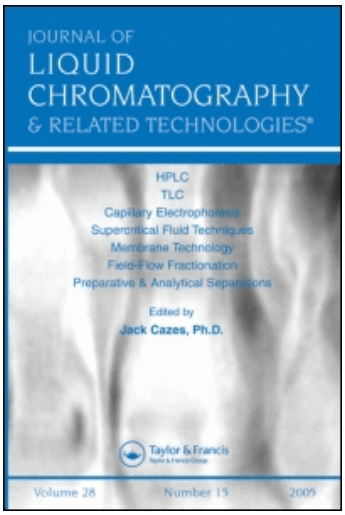


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# Determination of Novel Plant Growth Promoting Diterpenes in *Callicarpa macrophylla* by HPLC and HPTLC

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**Abstract:** Methods based on HPTLC and RP-HPLC with UV detection for rapid quantitative determination of two major plant growth promoters in *Callicarpa macrophylla*, calliterpenone (**1**) and calliterpenone monoacetate (**2**) are described. The recoveries of the two compounds were between 97.5–100.8% by HPTLC method and 99.3–100.9% by HPLC assay. The relative standard deviations of the two compounds ranged between 1.26–1.68 (Intra-day) and 1.06–1.68 (Inter-day) for HPTLC and 0.02–0.92 (Intra-day) and 0.03–0.92 (Inter-day) for HPLC. The methods were used for routine analysis of two compounds in the leaves of the plant.

**Keywords:** *Callicarpa macrophylla*, Calliterpenone, Calliterpenone mono acetate, diterpenes, Method comparison

## INTRODUCTION

Plant growth promoters are of commercial importance in intensive agriculture and agri-business of high value crops for their organic cultivation. Compounds like brassinosteroids (BRs), aurines, cytokinins, gibberellins

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and abscisic acid are important compounds of plant growth promoting group of chemicals.[1–3]

About twenty species from Callicarpa genus are distributed in China and South Asia.[4] *Callicarpa macrophylla* vahl (verbenaceae) is an erect shrub commonly found in the Indo-gangetic region and sub-Himalayan tracks of India upto an altitude of 2000 m.[5,6] Number of compounds such as amino acids, benzenoids, carbohydrates, lipids, numerous diterpenes, flavonoids, phenylpropanoids, phytosterols, sesquiterpenes, and triterpenes are reported from the genus Callicarpa.[7] Recently, we have reported *C. macrophylla* as a prominent source of plant growth promoting diterpenoids calliterpenone and its acetate.[8,9] Similar to that of abbeokutone (precursor of gibberellins), compounds calliterpenone (**1**) and calliterpenone monoacetate (**2**) resulted not only in significant plant growth promoting activities in mono and dicotyledonous plant species but were also found to antagonize the growth retardant effect of allelochemicals.[10,11]

Keeping in view the importance of plant as a promising and cost effective source of phyto-growth promoter, a rapid and sensitive analytical procedure is needed for the quality assessment of test materials. Although HPLC method was more precise and accurate, HPTLC may have capability of parallel chromatography because of its simplicity and economy of analysis. Our continued interest on developing rapid analytical techniques[12–18] resulted in a sensitive high performance liquid chromatography (HPLC) method for the quantitation of compounds (**1**) and (**2**) in *C. macrophylla*. In addition, a comparatively rapid HPTLC method, suitable for high throughput screening, has also been developed. To the best of our knowledge, no analytical method is available for quantitation of these valuable phytogrowth promoters.

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## EXPERIMENTAL

### Chemicals, Reagents and Plant Material

Standards (**1**) and (**2**) were isolated in the laboratory and structures (Fig. 1) were confirmed by spectral analysis.[19,20] All solvents and reagents used were of either analytical or HPLC grade (Merck, India) unless otherwise specified. Before use, the solvents were filtered through a 0.45 μm Millipore membrane (Millipore, Billerica, MA) after sonication for 15 min.

Leaves of *C. macrophylla* were collected from the plant grown in CIMAP experimental farm of the Institute at Lucknow. Herbarium specimen and seeds of above test plant species are available in the National Gene Bank of Medicinal and Aromatic Plants at CIMAP, Lucknow.

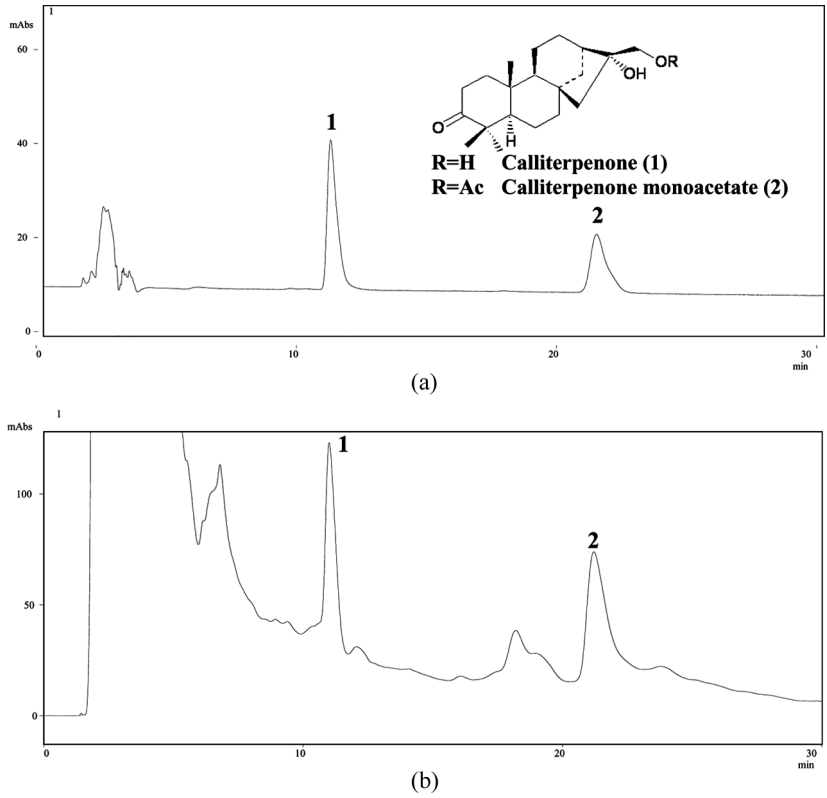


***Figure 1.*** HPLC chromatograms (a) artificial mixer of standard compounds **1** and **2** (b) methanol extract of *C. macrophylla* leaves.

## Standard Solution and Sample Preparation

Ten milligram of each standard was placed in a 10 mL volumetric flask and dissolved in methanol (stock solution). Working stocks for calibration curve were prepared by dilution. Samples of dried and finally powdered leaves (1.0 g) were sonicated in 10 mL methanol for 30 min followed by centrifugation for 10 min at 10,000 rpm. The supernatant was transferred to a flask. The procedure was repeated three times and the pooled extract was concentrated under vacuum, re-dissolved in 1.0 mL methanol for HPLC and 5.0 mL methanol for HPTLC analysis.

## Equipment

HPLC experiments were performed using a LC-10A HPLC system (Shimadzu, Japan) equipped with two LC-10A pumps controlled by a

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***Table 1.*** Overview of method development for the quantitation of calliterpenone (**1**) and calliterpenone mono acetate (**2**) in *C. macrophylla*

| Parameters | **1** | **2** |
|---|---|---|
| HPLC method | | |
| Working concentration range | 0.25–1.0 mg/mL | 0.25–1.0 mg/mL |
| Slope | 83007 | 80545 |
| Intercept | 12465 | 41696 |
| Correlation coefficient | 0.9998 | 0.9999 |
| Limit of detection (LOD) (μg) | 0.21 | 0.08 |
| Limit of qunatitation (LOQ) (μg) | 0.70 | 0.25 |
| *System suitability* | | |
| K′ | 6.26 | 12.98 |
| Separation factor | 5.38 | 2.38 |
| Capacity factor | 51.53 | 10.24 |
| Tailing factor, | 1.67 | 1.55 |
| Resolution factor | 13.36 | 2.38 |
| *Specificity* | | |
| Peak purity | | |
| Up | 0.999 | 0.999 |
| Down | 0.999 | 0.999 |
| UV-Vis spectra matching | | |
| Up | 0.66 | 0.41 |
| Apex | 2.68 | 0.95 |
| Down | 1.08 | 0.46 |
| HPTLC method | | |
| Working concentration range | 1–5 μg/spot | 1–5 μg/spot |
| Slope | 5514.0 | 1770.5 |
| Intercept | 3803.3 | 34.8 |
| Correlation coefficient | 0.9905 | 0.9888 |
| Limit of detection (LOD) (μg) | 0.23 | 0.22 |
| Limit of qunatitation (LOQ) (μg) | 0.78 | 0.73 |
| *Specificity* | | |
| Peak purity | | |
| R (s,m)[a] | | |
| Standard track (Reference compound) | 0.9999 | 0.9999 |
| Sample track (Methanol extract) | 0.9999 | 0.9998 |
| R (m,e)[b] | | |
| Standard track (Reference compound) | 0.9999 | 0.9999 |
| Sample track (Methanol extract) | 0.9998 | 0.9998 |

[a]Correlation of spectrum at start of peak with spectrum at the centre of peak at 610 nm scanning.
[b]Correlation of spectrum at center of peak with spectrum at the end of peak at 610 nm scanning.

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CBM-10 interface module, SIL-10 ADVP autoinjector and SPD-M 10Avp photodiode array detector. Data were collected and analyzed using a class LC-10 work station. Before use, solvent were filtered by a Millipore (Bed ford, MA, USA) filtering unit (0.45 μm). HPLC column used was Waters Spherisorb ODS 2 (250 × 4.6 mm id, 10 μm). Automatic TLC Sampler (ATS-4), Vario system, Immersion device III, TLC plate heater, TLC scanner WinCats-III, Reprostar 3 (All CAMAG, Muttnez, Switzerland) were used during the HPTLC method development.

### Validation of HPLC and HPTLC Methods

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The calibration curves were constructed by plotting the peak area *vs* concentration of growth promoters **1** and **2** and the linear regression equations were prepared using least-square method. Calibration curves were linear in the working concentration range. The limit of detection (LOD), calculated as the amount of analyte required to obtain a signal-to-noise ratio of 3, while the limit of quantitation (LOQ) was considered for signal-to-noise ratio of 10 (Table 1). Both HPLC and HPTLC methods were validated following the International guidelines for parameters like linearity, precision, accuracy, specificity and recovery[21,22] using adequate statistical estimates (%RSD, least square regression and residual analysis).[23] Recovery was carried out by spiking specified amount of test compounds **1** and **2**. Robustness of both the developed HPLC and HPTLC methods were evaluated by observing influence of small deliberate changes in the chromatographic parameters which may affect performance of the method. The coefficient of variation of peak areas was calculated for each parameter. Intermediate precision was calculated as intra-day and inter-day precision. System suitability was recorded as parameters like capacity factor, tailing factor and number of theoretical plates (Table 1).

### Statistical Analysis

Data analysis was carried out using XLSTAT 2008.5 software. Correlation coefficient using Least Square method and ANOVA shows similarities between samples analyzed by two methods.

## RESULTS AND DISCUSSION

### Optimization of HPLC Conditions and Method Validation

A representative HPLC separation for **1** and **2** in artificial mixture and sample extract are presented (Fig. 1). Chromatographic conditions

were optimized for a better separation of compounds **1** and **2** considering the parameters such as mobile phase, detection wavelength, column type and extraction solvent. A clear and good separation of the peaks corresponding to compounds **1** and **2** was achieved using mobile solvent methanol-water (45:55, v/v), flow rate 1 mL/min., detection wavelength 210 nm at retention times 11.03 and 21.26 min, respectively. Method repeatability (intra- and inter-day assay) was evaluated by estimating the corresponding response in triplicate on the same day and on three different days. The relative standard deviation (RSD%) of the estimates are summarized in Table 2. %RSD in inter- and intra-day assay for both the compounds **1** and **2** were <5%. The accuracy of the method was determined by recovery tests performed by adding three different concentrations of standards **1** and **2** to leaf extract. Spiked samples were then subjected to the entire procedure as above. The results showed recoveries 99.3–100.7 and 98.9–100.9, respectively of the HPLC analysis of compounds **1** and **2**. The peaks were confirmed by comparing $R_t$, peak purity and absorption spectra of **1** and **2** in standard and sample injections. The robustness of method was determined by measuring the effect of small and deliberate changes in the analytical parameters on retention time and peak area counts. The parameters that were taken into consideration were mobile phase composition, flow rate and temperature. At a time only one parameter was changed while others were kept constant. The standard deviations (%RSD) of retention time and peak area counts were calculated for each parameter and %RSD values were found to be in the agreement to the robustness of method (Table 4).

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## Optimization of HPTLC Conditions and Method Validation

A Vario system was used to optimize the mobile solvent by trying different ratio of solvents of varying polarity using silica gel $60F_{254}$ TLC plate (Merck Cat # 1.05729.001). A well resolved separation was achieved by using the mobile solvent ethyl acetate: hexane (55:45, v/v). Spots were applies as band of 6.0 nm, distance between band 15.0 mm, distance from the edge 15.0 mm and distance from the bottom of the plate 15.0 mm using Linomat IV spotter with a speed of 8 µL/min. The plates were developed in ascending mode for a distance of 9.0 cm in a vertical twin trough chamber, previously saturated for 2.0 min with the mobile solvent, under the laboratory condition (temperature $25 \pm 2°C$ and relative humidity 35–40%). After TLC run, the air dried plates were immersed (dipping time 2 s, dipping speed 5 cm/sec) in freshly prepared vanillin – sulphuric acid derivatizing reagent (vanillin:ethanol:$H_2SO_4$–1 g:95 mL: 5 mL) followed by heating at 110°C for 15 min. The densitometric scanning was performed in the reflectance/absorbance mode, slit width

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***Table 2.*** Intra- and inter-day precision of HPLC and HPTLC methods

| | | Intra-day | | | | Inter-day | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Calliterpenone | | Calliterpenone monoacetate | | Calliterpenone | | Calliterpenone monoacetate | |
| Calliterpenone (μg) | Calliterpenone monoacetate (μg) | Mean μg (n = 5) | RSD (%) | Mean μg (n = 5) | RSD (%) | Mean μg (n = 3) | RSD (%) | Mean μg (n = 3) | RSD (%) |
| HPLC Method | | | | | | | | | |
| 3.0000 | 3.0000 | 3.0211 | 0.02 | 3.0121 | 0.08 | 3.0324 | 0.56 | 3.0020 | 0.42 |
| 6.0000 | 6.0000 | 5.9240 | 0.85 | 5.9840 | 0.92 | 6.0216 | 0.03 | 6.1214 | 0.84 |
| 9.0000 | 9.0000 | 9.1031 | 0.31 | 8.9640 | 0.61 | 8.321 | 0.18 | 8.9823 | 0.92 |
| HPTLC Method | | | | | | | | | |
| 3.0000 | 3.0000 | 2.9462 | 1.28 | 2.9682 | 1.48 | 2.8924 | 1.06 | 3.0121 | 1.08 |
| 6.0000 | 6.0000 | 5.8540 | 1.32 | 6.0124 | 1.26 | 5.9080 | 1.31 | 6.0141 | 1.26 |
| 9.0000 | 9.0000 | 8.9860 | 1.58 | 8.6740 | 1.68 | 9.0124 | 1.68 | 8.6820 | 1.68 |

***Table 3.*** Recovery study (n = 3)

| Amount of calliterpenone added (μg) | Amount of calliterpenone recovered (μg) | Recovery (%) | Amount of calliterpenone mono acetate added (μg) | Amount of calliterpenone mono acetate added (μg) | Recovery (%) |
|---|---|---|---|---|---|
| HPLC Method | | | | | |
| 5.00 | 4.96 | 99.3 | 5.00 | 4.94 | 98.90 |
| 10.00 | 10.08 | 100.9 | 10.00 | 10.02 | 100.19 |
| 15.00 | 15.09 | 100.7 | 15.00 | 15.13 | 100.90 |
| HPTLC Method | | | | | |
| 3.00 | 2.97 | 99.2 | 3.00 | 2.97 | 97.20 |
| 6.00 | 6.02 | 100.4 | 6.00 | 6.02 | 100.83 |
| 9.00 | 8.77 | 97.5 | 9.00 | 8.77 | 98.48 |

6.00 mm × 0.40 mm, scanning speed 20 mm/sec and data resolution 10 mm/step, detection wave length 610 nm. Quantitation was performed using calibration curve. The satisfactory resolutions of the components in plant sample (Fig. 2) with pure peak (Table 1) were obtained.

***Table 4.*** Results of robustness data

| | Variations (%RSD) | | | |
|---|---|---|---|---|
| | Calliterpenone (**1**) | | Calliterpenone mono acetate (**2**) | |
| Parameters | Retention[a/b] | Peak area | Retention[a/b] | Peak area |
| *HPLC analysis* | | | | |
| Mobile phase composition | 0.24 | 0.85 | 0.22 | 0.85 |
| Flow rate | 0.12 | 2.02 | 0.12 | 2.02 |
| Column temperature | 0.19 | 2.12 | 0.17 | 2.12 |
| *HPTLC analysis* | | | | |
| Mobile phase composition | 0.15 | 0.46 | 0.11 | 0.86 |
| Time gap between spotting and plate development | 0.11 | 0.21 | 0.12 | 0.26 |
| Derivatization time (plate heating time) | 0.19 | 2.87 | 0.12 | 2.47 |
| Time gap between derivatization and scanning | 0.15 | 2.88 | 0.14 | 2.66 |

[a]Retention time in HPLC analysis.
[b]Retention factor in HPTLC analysis.

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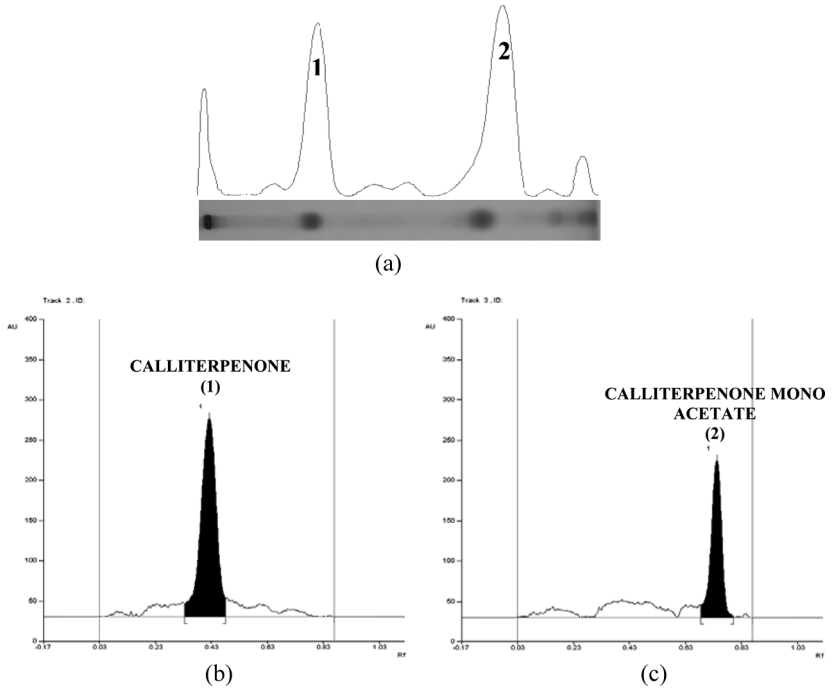


***Figure 2.*** HPTLC analysis of growth promoters (a) fingerprint of methanol extract of *C. macrophylla* after derivatization with vanillin-sulphuric acid reagent (b) densitogram of standard of calliterpenone and (c) standard of calliterpenone mono acetate at 610 nm.

The calibration curves for **1** and **2** were plotted with five different concentrations by plotting the peak area versus concentrations in working concentration range (Table 1). Good linearity (coefficient of determination $r^2 > 0.99$) was achieved in the investigated range for **1** and **2**. A statistical residual plot analysis also demonstrated that residuals were randomly distributed around the zero value. This confirmed the choice of the linear fit model. The limit of detection and limit of quantification of this method were also determined as S/N ratio 3 and 10, respectively (Table 1). For accuracy determination known amounts of **1** and **2** in three concentration ranges (3, 6 and 9 μg/spot) were added to pre quantified extract of plant sample. Recoveries obtained were 97.5–100.4 and 97.2–100.8 for compounds **1** and **2**, respectively (Table 3). The intra-day and inter-day RSD values ranged for **1** and **2** were 1.28–1.58, 1.26–1.68 and 1.06–1.68, 1.08–1.68, respectively. The band for reference compounds **1** and **2** in sample were confirmed by comparing the corresponding $R_f$ (0.43, 0.73) and absorption spectra of the

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bands corresponding to that of standards **1** and **2**. The robustness (Table 4) was found within ICH limit when scanned at 610 nm but compromised (higher %RSD value) after derivatization.

## Correlation and Comparison of Two Chromatographic Methods

The concentrations of calliterpenone (**1**) and calliterpenone monoacetate (**2**) obtained by HPTLC were in a range similar to that obtained by HPLC. The correlations of both methods were in a good agreement ($r^2$ between 0.9955 and 0.9992, (Fig. 3). The costs and analysis time comparison was performed which restricted on the running costs due to the study design using the same plant material, the same sample preparation, but two different chromatographic methods. The cost of stationary phase is much less in HPTLC than HPLC. The costs for HPTLC

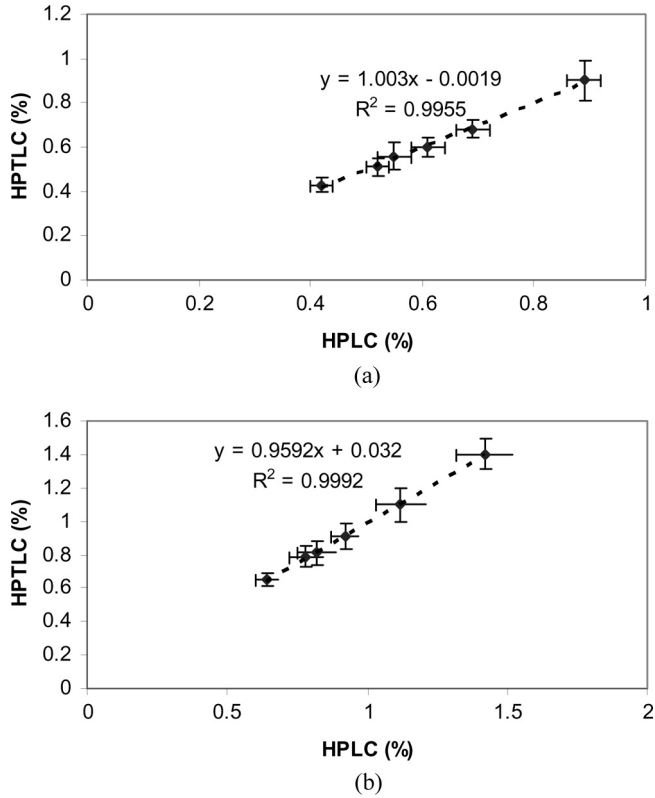


***Figure 3.*** HPLC/HPTLC correlation for calliterpenone (a) and calliterpenone mono acetate (b) content in six different plant samples of *C. macrophylla*.

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***Table 5.*** Calliterpenone and calliterpenone mono acetate content (on plant dry weight basis) in *C. macrophylla* by HPLC and HPTLC methods (n = 3)

| | HPLC method | | HPTLC method | |
|---|---|---|---|---|
| Samples | Calliterpenone (%) (mean ± sd) | Calliterpenone mono acetate (%) (mean ± sd) | Calliterpenone (%) (mean ± sd) | Calliterpenone mono acetate (%) (mean ± sd) |
| AK-20 | 0.52 ± 0.02 | 0.82 ± 0.07 | 0.51 ± 0.04 | 0.81 ± 0.07 |
| AK-21 | 0.55 ± 0.03 | 0.78 ± 0.06 | 0.56 ± 0.06 | 0.79 ± 0.06 |
| AK-22 | 0.61 ± 0.03 | 0.92 ± 0.05 | 0.60 ± 0.04 | 0.91 ± 0.08 |
| AK-23 | 0.69 ± 0.03 | 1.12 ± 0.09 | 0.68 ± 0.04 | 1.10 ± 0.10 |
| AK-26 | 0.89 ± 0.03 | 1.42 ± 0.10 | 0.90 ± 0.09 | 1.40 ± 0.09 |
| AK-27 | 0.42 ± 0.02 | 0.64 ± 0.04 | 0.43 ± 0.03 | 0.65 ± 0.04 |

analysis of six plant samples are about a factor of 5 lower than the costs for HPLC analysis. However, due to simultaneous analysis of samples, the HPTLC method is about 7 times faster than HPLC.

## Method Application

The developed and validated method was applied for **1** and **2** determination in the leaf of *C. macrophylla*. The content of calliterpenone and

***Table 6.*** Effect of collection time on calliterpenone and calliterpenone mono acetate content in *C. macrophylla*

| | Content (%) dry weight basis | | | | | |
|---|---|---|---|---|---|---|
| | Calliterpenone | | | Calliterpenone mono acetate | | |
| Month of collection | Lower leaves | Middle leaves | Top leaves | Lower leaves | Middle leaves | Top leaves |
| Feb | 0.230 | 0.423 | 0.610 | 0.356 | 0.568 | 0.856 |
| March | 0.320 | 0.385 | 0.550 | 0.456 | 0.652 | 0.758 |
| April | 0.389 | 0.495 | 0.620 | 0.523 | 0.720 | 0.723 |
| May | 0.540 | 0.627 | 0.700 | 0.702 | 0.850 | 0.856 |
| June | 0.586 | 0.785 | 0.900 | 0.796 | 0.996 | 0.850 |
| July | 0.664 | 0.892 | 1.203 | 0.856 | 1.025 | 1.421 |
| August | 0.523 | 0.652 | 0.988 | 0.752 | 0.952 | 1.256 |
| September | 0.304 | 0.523 | 0.892 | 0.456 | 0.825 | 1.102 |
| October | 0.256 | 0.345 | 0.785 | 0.385 | 0.723 | 0.998 |
| November | 0.156 | 0.256 | 0.562 | 0.289 | 0.456 | 0.756 |
| December | 0.098 | 0.125 | 0.321 | 0.158 | 0.256 | 0.623 |

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calliterpenone mono acetate in six different plant samples are summarized in Table 5. Retention times and UV spectra of respective compounds were used to ensure the identity of the compounds **1** and **2** in the samples. Results are comparable and the method may be applied for qualitative and quantitative evaluation of *C. macrophylla* for the growth promoters **1** and **2**. Calliterpenone content varies from 0.4–0.9% where as its monoacetate varies for 0.6–1.4% on plant dry weight basis. The HPLC method, was also applied to access the quality of different germplasm, their collection time variability and distribution in different leaves for chemicals **1** and **2** (Table 6). The climate conditions are affecting significantly the content of **1** and **2** in different leaves with highest in top leaves in the month of July.

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## CONCLUSION

The present paper deals with the development of both HPLC and HPTLC methods for quantitation of plant growth promoters **1** and **2**. Methods have been validated as per ICH guidelines. Being more accurate, the present LC method is suitable for precise analysis whereas HPTLC method is suitable for rapid screening of plant samples because of its simplicity and low operating cost.

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