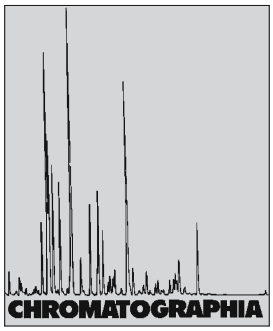
# Quantitative TLC Analysis of Sterol (24$\beta$-Ethylcholesta-5,22*E*,25-triene-3$\beta$-ol) in Agnimantha (*Clerodendrum phlomidis* Linn)

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## Abstract

A quantitative method using silica gel 60$F_{254}$ high performance thin layer chromatography plates, automated bandwise sample application, and automated visible mode densitometric method has been developed for the determination of 24$\beta$-ethylcholesta-5,22*E*,25-triene-3$\beta$-ol (ECTO) in the aerial part of *Clerodendrum phlomidis*. ECTO was used as a chemical marker for the standardization of *C. phlomidis* plant extracts. The separation was performed on silica gel 60$F_{254}$ TLC plates using chloroform-methanol (98.5: 1.5, *v/v*) as mobile phase. The quantitation of ECTO was carried out using the densitometric reflection/absorption mode at 650 nm after post chromatographic derivatization with anisaldehyde reagent. A precise and accurate quantification can be performed in the linear working concentration range of 150–400 ng band$^{-1}$ with good correlation ($r^2$ = 0.996). The method was validated for peak purities, precision, robustness, limit of detection (LOD) and quantitation (LOQ), etc. as per ICH guidelines.

## Keywords

Thin layer chromatography
Fingerprinting
Sterol
*Clerodendrum phlomidis*

## Introduction

*Clerodendrum phlomidis* Linn (family: *Verbenaceae*), is a large, often rambling shrub which is distributed widely throughout India [1]. *C. phlomidis* is one of the components of *Dasmula*, a well known ayurvedic formulation used in the treatment of fatigue of women after child birth. *Clerodendrum* species have been used in Indian and Chinese traditional medicine for ancient times. A decoction of *C. phlomidis* leaves is used along with other plants for inflammation, and is effective in treating bronchitis, headache, weakness, drowsiness and digestive problems [2]. The plant is reported to possess anti-fungal, anti-pyretic activity [3] and also inhibits several plant and human pathogenic fungi [4]. In addition anthelmitic [5], antimicrobial [6], hypoglycemic [7], and antidiarrhoeal activities [8] were also reported. Isolation and characterization of phytochemicals of *C. phlomidis* have been reviewed [9].

The genus *Clerodendrum* is known to be a source of rare sterols [10–12]. *C. phlomidis* like many other species is a rich source of sterols having the same skeleton [9]. Sensitive and reliable methods are required to establish the quality of the herbs and their derived products. Although, *C. phlomidis* is an important plant used in Indian and Chinese system of medicine, assay of these phytosterols has been hampered by lack of analytical methods for reproducible and accurate determination of their concentrations in raw herbs as well as their products. The literature survey revealed that no


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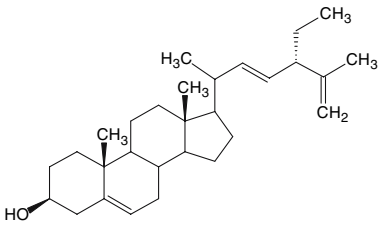


(24*S*)-Ethylcholesta-5,22,25-trien-3β-ol (**ECTO**)

**Fig. 1.** Structure of chemical marker of *C. phlomidis*

**Table 1.** Extractability of different solvent for marker compounds ECTO from the aerial parts of *C. phlomidis*

| Extraction solvents | Amount of compound quantified[a] (% *w*/*w*) |
|---|---|
| Hexane | 0.0552 |
| Chloroform | 0.0547 |
| Ethanol (95%) | 0.0338 |
| Methanol | 0.0927 |
| Ethyl acetate | 0.1047 |

[a] Plant dry wt. basis

analytical procedure is reported for the quality assay of the Agnimantha (*C. phlomidis*). Recently, a chemical marker based quantitative TLC method for other *Clerodendrum* species, i.e. *C. inermis* has been reported [13]. Mainly GLC and LC methods are used for determination of sterol analysis [14–16], but they suffer from various drawback such as complex derivatization steps and/or need of specific detectors such as ELSD or mass. A TLC quantitative method for ergosterol from fungi in soil has been reported using fluorodensitometry which suffers with sterol decomposition on TLC plates at low concentration in a short duration [17]. Progress in instrumentation of TLC analysis led this technique to be simple more efficient and reliable [18]. In this paper we are reporting a new validated TLC method for the quantification of marker sterol, 24β-ethylcholesta-5,22*E*,25-triene-3β-ol in *C. phlomidis*.

# Experimental

## Plant Materials

Aerial parts of *C. phlomidis* were collected locally from Lucknow and identified by the Botany and Pharmacognosy department of our institute. A voucher specimen (CIMAP No 9054) has been deposited in the herbarium of the institute.

## Chemicals and Standard

All the solvents used were either of analytical or LC grade and purchased from E Merck (Mumbai, India). Before use, the solvents were filtered through a 0.45 μm Millipore membrane (Millipore, Billerica, MA, USA). Precoated silica gel $60F_{254}$ TLC plates (10 × 10 cm; 20 × 10 cm; E Merck, Darmstadt, Germany) were used for optimization of the analytical method. The marker compound, 24β-ethylcholesta-5,22*E*,25-triene-3β-ol (ECTO) was isolated (purity > 99%) and characterized by spectral analysis in our laboratory.

## Extraction and Isolation of ECTO from *C. phlomidis*

Steroid (ECTO, Fig. 1) was extracted from *C. phlomidis* and confirmed as reported earlier [19, 20].

## Standard Stock Solution and Sample Preparation

Standard stock solutions containing 1.0 mg $mL^{-1}$ of the marker compound ECTO was prepared in methanol and filtered through 0.45 μm filters. Working stocks for calibration studies were prepared by dilution. Plant extracts were prepared from dried and powdered aerial parts (250 mg) of *C. phlomidis*. Various solvents from low polarity to high polarity viz. *n*-hexane, chloroform, ethyl acetate, methanol and ethanol were used separately to prepare individual extracts. Dried and powdered aerial parts (250 mg) of *C. phlomidis* was soaked with *n*-hexane at room temperature (5 mL, 10 h contact time) and filtered. The process of extraction was repeated three times to avoid incomplete extraction. Combined extracts were filtered, dried under vacuum and made up to 1 mL with *n*-hexane and centrifuged at 10,000 rpm for 10 min. The supernatants were used for TLC analysis. Similarly other extracts were prepared in chloroform, ethyl acetate, methanol and ethanol for TLC analysis.

## Apparatus

Automatic TLC Sampler (ATS-4), Vario system, Immersion device III, TLC plate heater, TLC scanner with WinCats software, Reprostar 3 (All CAMAG, Muttenz, Switzerland) and Spinwin (Tarson, India) were used during the work.

## Chromatographic Experiments

Precoated TLC silica gel $60F_{254}$ plates were activated at 60 °C for 5 min prior to chromatography. Standard and sample solutions were spotted in the form of bands of width 5 mm at 15 mm from both the lower and left edge, and 20 mm space between two bands, with a microliter syringe using automatic TLC sampler, under continuous nitrogen current. A constant application rate of 5 μL $s^{-1}$ was employed. Linear ascending development was carried out in twin trough glass chambers 10 × 10 cm or 20 × 20 cm saturated with the mobile phase. The mobile phase selection was carried out using the Vario System wherein different compositions consisting of different ratios of solvents of varying polarity with all three stationary phases were tried. Finally, a mobile phase consisting of chloroform-methanol (98.5: 1.5, *v*/*v*) was found suitable for satisfactory separation and quantitation of ECTO with interfering components of sample matrix. Saturation time of TLC chamber in the mobile phase was optimized to 2 min for better resolution. Detection of the spots was carried out by dipping the chromatographic plates in a freshly prepared anisaldehyde: sulfuric acid: acetic acid (1 mL: 5 mL: 95 mL) reagent using Immersion device and subsequent heating at 110 °C for 15 min on TLC plate heater. TLC Scanner-III fitted with winCATS 1.3.3 software was

applied for quantitative evaluation. A TLC plate was scanned at different wavelength to select the scanning wavelength 650 nm after derivatization. The densitometry scanning was performed in the reflectance/absorbance mode at 650 nm, slit width $6.00 \times 0.40$ mm, scanning speed 20 mm $s^{-1}$ and data resolution 10 μm $step^{-1}$. Savitsky-Golay 7 was used for data filtering and the lowest slope for baseline correction in order to integrate the area. For recording of characteristic UV absorption spectra (200–800 nm) of sample track, both deuterium and tungsten lamps were used, while for derivatized band of compounds ECTO and sample tracks in the range 400–800 nm, tungsten lamp was used. Reprostar 3 with cabinet cover and mounted digital camera was used for imaging and archiving the thin layer chromatograms. Concentrations of the compounds chromatographed were determined from the intensity of the reflected light. Evaluation was via peak areas with linear regression.

**Table 2.** Overview of method parameters for the quantitation of ECTO in *C. phlomidis*

| Parameters | ECTO |
|---|---|
| Retention factor ($R_f$) | 0.54 ± 0.05 |
| Linearity | |
| Working concentration range | 25–200 μg $mL^{-1}$ |
| Regression equation[a] | $Y$ = (11.24 ± 0.19) $X$ + (679.20 ± 45.91) |
| Correlation coefficient ($r^2$) | 0.9964 |
| Goodness of fit ($S_{yx}$) | 92.58 |
| Sensitivities | |
| Limit of detection (LOD) | 24.71 ng |
| Limit of quantitation (LOQ) | 82.28 ng |
| Specificity | |
| Peak purity | |
| $R$ (s,m)[b] | |
| Standard track (ECTO) | 0.9996 |
| Sample track (EtOAc extract) | 0.9995 |
| $R$ (m,e)[c] | |
| Standard track (ECTO) | 0.9991 |
| Sample track (EtOAc extract) | 0.9988 |
| Precision and accuracy[d] | |
| Instrumental (% RSD) | 0.89 |
| Intra-day[d] (% RSD) | 1.21 |
| Inter-day[d] (% RSD) | 1.86 |

[a] Five data point each in triplicate; $X$, amount of compound (ng $band^{-1}$); $Y$ peak area in AU
[b] Correlation of spectrum at start of peak with spectrum at the center of peak
[c] Correlation of spectrum at center of peak with spectrum at the end of peak
[d] $n = 5$

**Table 3.** Results of recovery study

| ECTO in sample (ng $band^{-1}$) | Spiked amount (ng $band^{-1}$) | Theoretical value (ng $band^{-1}$) | Experimental value (ng $band^{-1}$) | Recovery (%) | Average recoveries (%) | RSD (%, $n = 3$) |
|---|---|---|---|---|---|---|
| 50.75 | 50 | 100.75 | 104.83 | 104.05 | 102.60 | 1.55 |
| | | | 103.55 | 102.78 | | |
| | | | 101.72 | 100.96 | | |
| | 100 | 150.75 | 155.50 | 103.15 | 102.44 | 4.90 |
| | | | 161.22 | 106.95 | | |
| | | | 146.56 | 97.22 | | |
| | 300 | 350.75 | 382.94 | 109.18 | 104.21 | 6.58 |
| | | | 374.23 | 106.69 | | |
| | | | 339.34 | 96.75 | | |

## Method Validation

Validation of the quantitative TLC method includes the evaluation of following performance parameters such as linearity, limit of sensitivities, specificity, precision and accuracy, recovery and robustness according to the ICH guidelines [21, 22].

## Linearity and Quantification

Working stock solutions were prepared by dilution to give solutions containing ECTO in the concentrations of 25, 50, 100, 150, and 200 μg $mL^{-1}$. Two microliter of each standard solution was spotted on the TLC plate to obtain absolute amounts of 50, 100, 200, 300 and 400 ng $band^{-1}$. 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 (ng $band^{-1}$) and dependent variable ($Y$) the peak area of ECTO. Regression analyses test of the compound was performed by Graph PAD Prism 3.0. The curves confirm the significant linear relationship between the concentration and the peak area (Table 2).

Two microliter of each sample solution from different locations 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 percent content of analyte in the sample. The results are presented in Table 1.

### *Limit of Detection and Quantification*

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated for ECTO using the linear regression equation (Table 2). 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

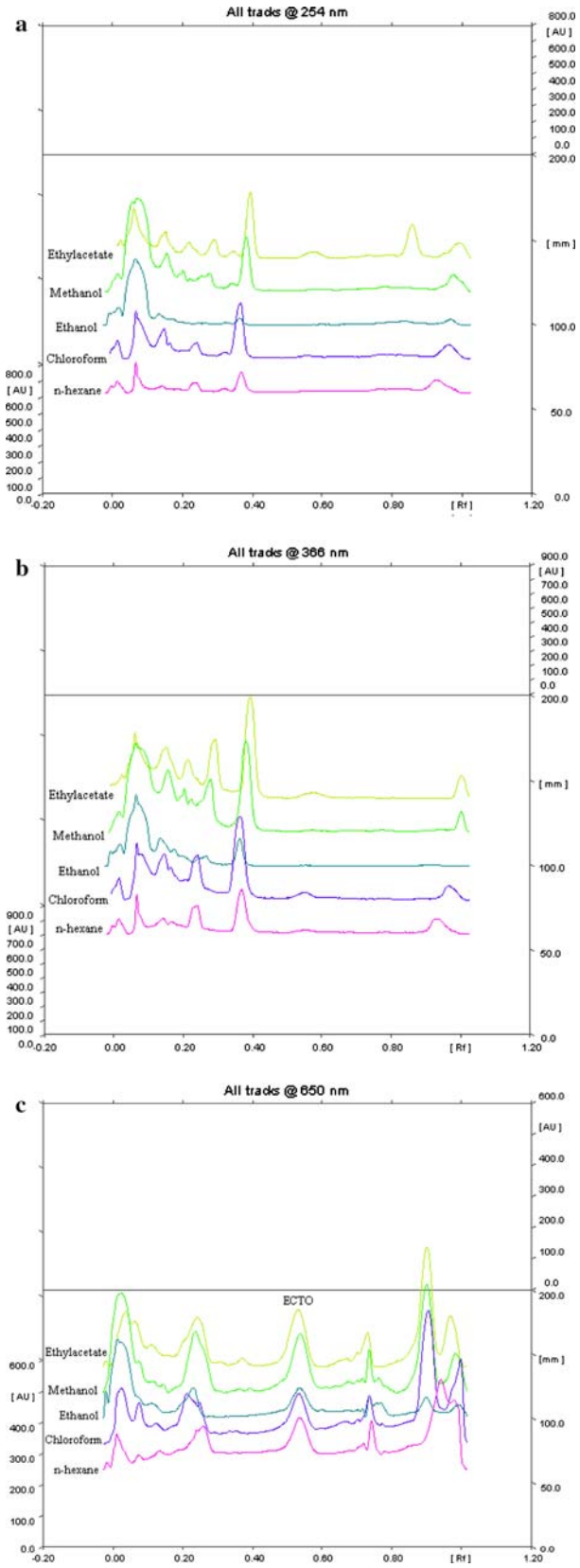


**Fig. 2.** TLC peak profile of various extracts scanned at 254 nm (**a**), 366 nm, (**b**) and (**c**) 650 nm after derivatization with anisaldehyde sulfuric acid reagent

sample. The band for ECTO in sample was confirmed by comparing the $R_f$ (0.54) and absorption spectra of the spot to that of standard. The peak purity of ECTO 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. Good correlation was also obtained between standards and sample overlay spectra ($r^2 > 0.99$).

### *Precision and Accuracy*

The repeatability of measurement ($n = 5$) of peak area ECTO were expressed in terms of percent coefficient of variation (% RSD). The intra- and inter-day variation was also evaluated at two concentration levels: 100 and 200 ng band$^{-1}$. The results depicted in Table 2 showed that the method is accurate and precise for the analysis of ECTO in *C. phlomidis*.

### *Recovery*

The accuracy of quantitation was assessed in a recovery study. For this purpose, a sample with known ECTO content was diluted, applied in triplicate onto the plate (2 µL), and individually spiked with three different amounts of ECTO to reach a final content in the lower, middle and upper range of pseudo-linear calibration curve (Table 2). Three different spiking concentrations (25, 50, 150 µg mL$^{-1}$) of the standard stock solutions of ECTO were prepared to calculate recovery (Table 3).

### *Robustness*

The robustness of the method was determined by introducing small changes in certain chromatographic parameters. Mobile phase having chloroform-methanol (98.5: 1.5, *v*/*v*) were tried with variation of 0.5% *v*/*v* in each solvent. Time gap between spotting to chromatography, from chromatography to scanning and derivatization time (plate heating time) was varied from 0, 10, 30 min. Robustness was performed at two levels; 100 and 200 ng 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. The overall low values of % RSD as shown in Table 4 indicated the robustness of the method. Quantitation was not significantly effected by changing scanning wavelength ± 5 nm.

## Results and Discussion

### Sample Preparation and Peak Profiling

The effect of extracting solvents was studied with respect to the percent content of ECTO in *C. phlomidis*. Various extracts viz. *n*-hexane, chloroform, ethyl acetate, methanol and ethanol were chromatographed to evaluate the extraction efficiency as well as the interferences due to co-eluted compounds. All the sample tracks were scanned at 254 and 366 nm wavelength (Fig. 2a, b), in addition to derivatization with anisaldehyde reagent (Fig. 2c). It is clearly evident that no interfering compound eluted in the sample tracks to affect the quantitation of the targeted marker ECTO (Fig. 2). Ethaylacetate as extraction solvent was found to be the most suitable and exhaustive solvent for sample preparation (Table 1). A representative denstiogram of ethyl acetate extract along with standard ECTO ($R_f$ 0.54) is presented in Fig. 3.

### Optimization of Mobile Phase

The TLC procedure was optimized in view to making reproducible quantitation of ECTO in the plant extracts. We observed that if $R_f$ of ECTO is between 0.50 and 0.60 then there is no possibility of merging of closet components. The mobile phase consisting of chloroform-methanol (98.5: 1.5, *v*/*v*) has resulted into a sharp, symmetrical, well-resolved separation of ECTO in sample track ($R_f$ 0.54) (Fig. 3).

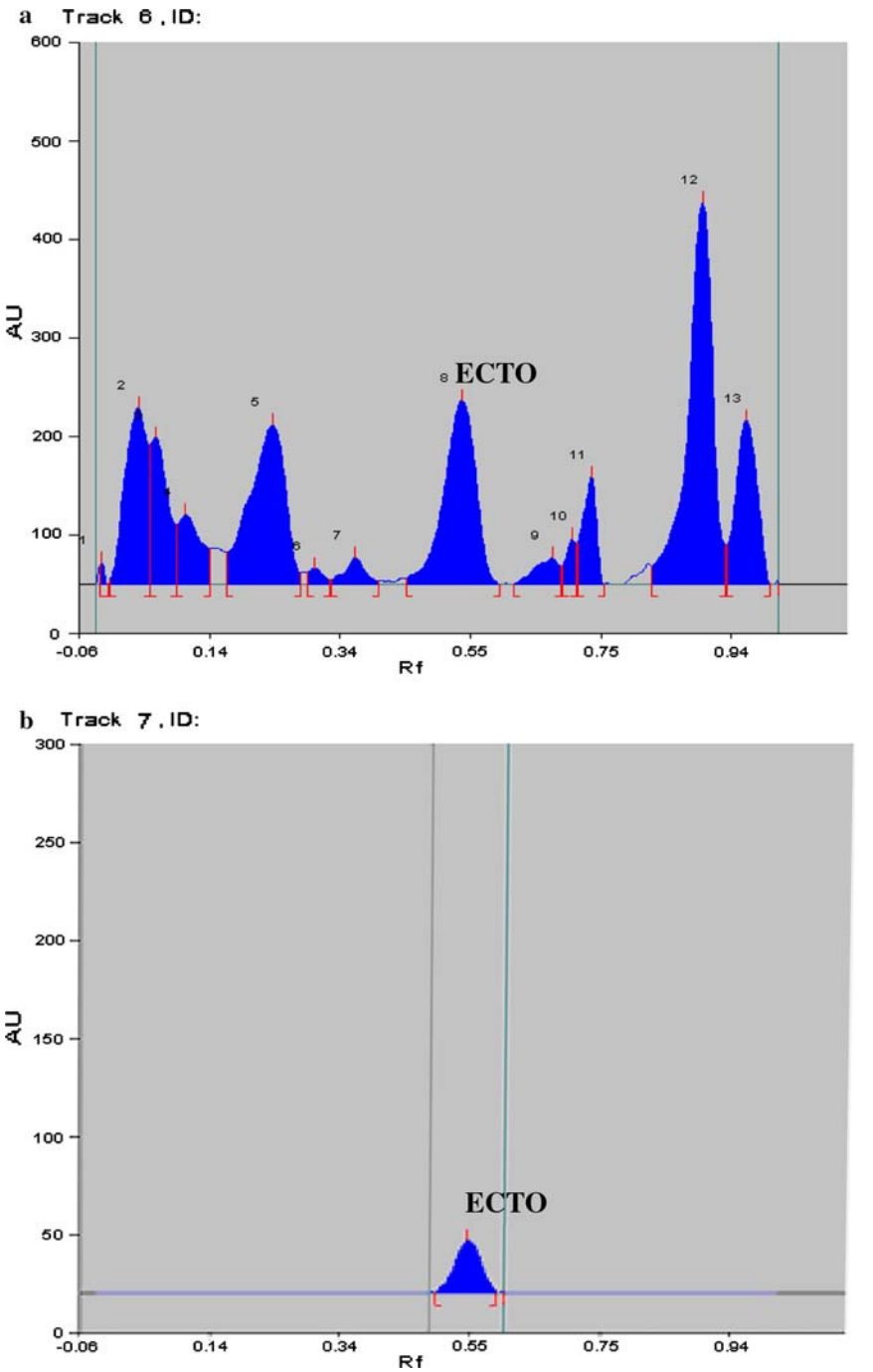


**Fig. 3.** Representative densitograms of **a** ethyl acetate extract of *C. phlomidis* (250 mg $mL^{-1}$) with **b** chemical marker ECTO (50 µg $mL^{-1}$) scanned at 650 nm after derivatization with anisaldehyde sulfuric acid reagent

### Quantitative Evaluation of ECTO in *C. phlomidis* and Fingerprinting

Three different samples of *C. phlomidis* were analyzed for its chemical marker, i.e. 24$\beta$-ethylcholesta-5,22*E*,25-triene-3$\beta$-ol (ECTO). On the basis of extraction efficiency ethyl acetate was selected for the quantitative analysis of ECTO. Well-separated bands of ECTO ($R_f$ 0.54) were observed in all the densitograms of the samples from different locations. There was no interference in the analysis of ECTO from the other closet components. The results are presented in Table 5. The ECTO content was source dependent. On critical examination of

**Table 4.** Robustness testing to access the stability of the TLC method ($n = 5$)

| Parameters | RSD (%) of peak area of ECTO |
|---|---|
| Mobile phase composition | 1.89 |
| Plate treatment | 1.55 |
| Time gap between spotting and plate development | 0.46 |
| Derivatization time (plate heating time) | 2.04 |
| Time gap between derivatization and scanning | 2.89 |

**Table 5.** Percent content of ECTO in *C. phlomidis* (calculated on plant dry weight basis)

| Locations | ECTO amount quantified (% *w*/*w*) |
|---|---|
| 1 | 0.105 ± 0.09 |
| 2 | 0.116 ± 0.08 |
| 3 | 0.127 ± 0.07 |

peak profiles of various extracts, we found that in addition to ECTO another compound ($R_f$ 0.39) was present in all five extracts, i.e. *n*-hexane, chloroform, ethanol, methanol, and ethyl acetate extracts, while compound $R_f$ 0.86 was present only in ethyl acetate extract. Another compound ($R_f$ 0.28) was present in all extracts except ethanol extract.

## Conclusion

Thin layer chromatography is a globally accepted practical solution to characterize the raw herbs, active constituent enriched extracts and their formulations. TLC method on silica gel $60F_{254}$ with chloroform-methanol (98.5: 1.5, *v*/*v*) and densitometric evaluation at 650 nm after anisaldehyde derivatization is simple, specific, precise, accurate and robust for the determination of 24$\beta$-ethylcholesta-5, 22*E*,25-triene-3$\beta$-ol (ECTO). This standardized TLC procedure may be used effectively for the screening analysis as well as quality evaluation of the plant or its derived herbal products.

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