

Research Article



HPTLC Estimation of Stigmasterol from Petroleum Ether Extract of Dried Leaf, Stem and Flower of *Ageratum Conyzoides* L

Anjoo Kamboj*, Ajay Kumar Saluja

Chandigarh College of Pharmacy, Landran, Mohali-140307 (Punjab), India.

*Corresponding author's E-mail: anjookamboj@gmail.com

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ABSTRACT

This study presents the first report of TLC densitometric method, which has been developed and validated for quantitation of stigmasterol from petroleum ether extract of dried leaf, stem and flower of *Ageratum conyzoides* using the solvent system of chloroform: ethanol (9.8:0.2, v/v). This method gave compact spots at $R_f = 0.45$ corresponding to stigmasterol. The method was validated using ICH guidelines in terms of precision, repeatability and accuracy. Linear range for stigmasterol was found to be 50-300 ng/spot and the leaf, stem and flower contents were found to be 0.1168 ± 0.05 , 0.289 ± 0.02 , and 0.0188 ± 0.01 %w/w, respectively. The limit of detection (LOD) and limit of quantification value for stigmasterol were found to be 14 and 45 μg , respectively. This simple, precise and accurate method gave good resolution from other constituents present in the extract. The method has been successfully applied in the analysis and routine quality control of herbal material and formulation containing *Ageratum conyzoides*.

Keywords: *Ageratum conyzoides*, densitometric method, HPTLC.

INTRODUCTION

Ageratum conyzoides Linn. (Asteraceae) is small, aromatic, annual herb, native to Tropical America, naturalized as a weed throughout India up to an altitude, 1800m, also found in the Andaman's. *Ageratum* is derived from the greek "a geras", meaning non-ageing, referring to the longevity of the flowers or the whole plant "conyzoides", is derived from "konyz", the greek name of *Inula helenium*, which it resembles. It is softly hairy, erect, branched, annual weed up to 80-90 cm in height. The leaves are stalked, ovate, opposite or upper ones alternate, more or less hairy on both sides, margins ciliated; flowers pale blue to purplish or white, pappus of 5-scales, dilated at the base almost equaling the corolla; fruits achenes, black in colour, 5- angled and attenuated at the base¹⁻³.

The roots, leaves, flowers and whole plants are of great medicinal value. A wide range of chemical compounds including alkaloids, cumarins, flavanoids, chromenes, benzofurans, sterols and terpenoids are present in this species. They are used for treatment of an array of ailments such as burns and wounds, for antimicrobial properties, for many infectious conditions and bacterial infections, arthrosis, headaches, dyspnea, pneumonia, analgesic, anti-inflammatory, antiasthmatic, antispasmodic and haemostatic effects, stomach ailments, gynecological diseases, leprosy and other skin diseases⁴⁻⁶.

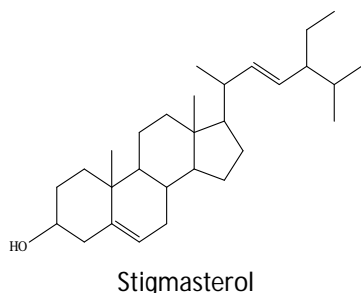
In the recent year advancement in of chromatographic and spectral fingerprints plays an important role in the quality control of complex herbal medicines⁷. HPTLC has become a routine analytical technique due to its advantages of reliability in quantification of analytes at micro and even in nanogram levels and cost effectiveness. It has proved a very useful technique because of its low

operating cost, high sample throughput and need for minimum sample clean-up. The major advantage of HPTLC is in reducing analysis time and cost per analysis. TLC has been known as the fast tool for the detection of compounds. Another advantage of TLC is the capability to detect more compounds than HPLC, although the resolution is poorer. In this regard, the compounds which cannot be eluted still can be detected. Moreover, the compounds having no UV absorption, e.g. sugar, still can be detected by reagent spraying. The TLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plants extracts. Thus, it can be used as a tool in the quality control in order to warranty that the active compounds are extracted. By means of data analysis system and optimized experimental conditions, HPTLC is also feasible for development of chromatographic fingerprint methods to determine and identify complex herbal extracts just like HPLC and GC. Furthermore, the colorful picture, like HPTLC image provides extra intuitive parameters of visible color and or fluorescence and unlike HPLC and GC, HPTLC can simultaneously determine different samples on the same plate. Such an approach causes the HPTLC method to maintain its innate advantage as well as get over a limitation of developing distance and plate efficiency.

Literature survey revealed that no method has been reported for quantitation of stigmasterol from leaves, stems and flower extracts of *Ageratum conyzoides*. Qualitative and quantitative standardization of stigmasterol was performed using HPTLC but not from this plant. Hence a densitometric HPTLC method has been developed in the present work for quantitation of stigmasterol from petroleum ether extract of dried leaf and stem *Ageratum conyzoides* which may useful to pharmaceutical industry for the authentication,



standardization and quantitation of stigmasterol component without being separated from the extracts⁹⁻¹³.



MATERIALS AND METHODS

Collection, Identification and Preparation of Plant Materials

The aerial parts of the plant were collected from Herbal Nature Park, Chuharpur, Yamuna Nagar, Haryana in the month September 2007. The plant was taxonomically identified, authenticated by Professor Dr. J. S. Sodhi, HOD, Botany Department, Guru Nanak Khalsa College, Yamuna Nagar, Kurukshetra University, Haryana and deposited with A. R. College of Pharmacy, Vallabh Vidya Nagar, Sardar Patel University, Gujrat. The aerial parts of the plant were manually separated was air dried, powdered, sieved, weighed and stored in air tight container and subsequently referred to as powdered drug.

Preparation of Standard Solutions

Preparation of stigmasterol (1mg/100ml) was prepared by transferring 1mg of stigmasterol accurately weighed into a 100 ml volumetric flask, dissolving in 50ml of chloroform. It was then sonicated for 10 min and the final volume of the solution was made up to 100ml with chloroform to get stock solution 10 µg/ml.

Preparation of Extracts

The dried powdered sample of leaf, stem and flower of *Ageratum conyzoides* (1g powdered) was refluxed on the water bath with 100ml of petroleum ether (60°-80°C) for 5-6 hours. The extract was concentrated under reduced pressure at 50°-60°C and finally made up to 10ml with HPLC grade chloroform and ready for HPTLC analysis and labeled as A5 for petroleum ether extract of leaf, A6 for petroleum ether extract of stem and A7 for petroleum ether extract of flower of *Ageratum conyzoides*.

Analytical Procedure

Chromatography Conditions

Chromatography was performed on a 10x10 cm preactivated HPTLC Silica gel 60 F₂₅₄ plates (Merck, Darmstadt, Germany). Aliquots of each of the extracts were separately applied (Samples and standard) to the plate as 6 mm wide band with an automatic TLC applicator Linomat-V with N₂ flow (CAMAG, Switzerland), 8 mm from the bottom. Densitometric scanning was performed on CAMAG scanner III at 490 nm. The plates were prewashed by methanol and activated at 60°C for 5

min prior to chromatography. The slit dimension was kept at 5.00 mm x 0.45 mm and 40 mm/sec scanning speed was employed. The mobile phase consisted of Chloroform: ethanol (9.8:0.2) and 10 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 10 x 10 cm twin glass chamber saturated with the mobile phase.

Detection and Quantitation

After sample application plates were developed in a Camag twin through glass tank pre-saturated with the mobile phase (10ml) Chloroform: Ethanol (9.8:0.2) for 20 minutes. The plate was developed in Camag horizontal developing chamber (10x10cm) at the room temperature up to 8cm. After development, plates were dried with a hair dryer and then derivatization of the chromatogram was performed by Camag glass reagent spray by spraying still hot plate with 5% sulphuric acid in methanol, heated at 105°C for 5min. Then plate was observed after 30 minutes under Camag UV cabinet (254 and 366nm). Quantitative analysis of the compounds was done by scanning the plates at 490nm using Camag TLC scanner III equipped with winCATS- V 1.2.3 software (Camag). The identification of stigmasterol was confirmed by superimposing the UV spectra of the samples and standards within same Rf 0.45 window.

A densitometry HPTLC analysis was also performed for the development of characteristic fingerprint profile, which may be used as marker for quality evaluation and standardization of the drugs.

Calibration Curve of Stigmasterol

The content of stigmasterol compound was determined by using a calibration curve established with standard concentration range from 50-300ng/spot. A stock solution of standard stigmasterol (10µg/ml) was prepared in chloroform. The different volume of stock solution 5, 10, 15, 20, 25 and 30µl were spotted on to HPTLC plate to obtained concentration 50, 100, 150, 200, 250 and 300 ng/spot respectively (band width 6mm, distance between the tracks mm) in triplicate on TLC plate using automatic sample spotter. Each concentration peak area was plotted against the concentration of stigmasterol spotted. The linear regression of standard curve was determined with $R^2 \pm SD = 0.999 \pm 0.26\%$. The linear regression line is $y = 11.163x + 690.892$ (Figure 2). The regression data has shown a good linear relationship over the concentration range of 50-300 ng/spot. The linearity of calibration graphs and adherence of the system to Beer's law are validated by high value of correlation coefficient and the SD for intercept value is noticed to be less than 2% (RSD 0.28%).

Method Validation

Precision

ICH guidelines were followed for the validation of analytical method developed for precision, repeatability and accuracy. Instrumental precision, intra-day precision

and inter-day precision of the method were determined. Instrumental precision were measured by replicate (n= 6) applications of same stigmasterol solution. Intra-day assay precision was evaluated by analysis of replicate (n=6) applications of freshly prepared standard solution of same concentration, on the same day. Intermediate precision was evaluated by analysis of replicate (n=6) applications of standard solution of the same concentration on six different days. Inter-and intra-day variation for determination of Stigmasterol was carried out at five different concentration levels of 50, 100, 150, 200 and 250 ng/spot. The repeatability of sample application and measurement of peak area have been expressed in terms of % CV and was observed to be <2% for Stigmasterol. The results show that there was no significant intra-and inter-day variation has been observed in the analysis of stigmasterol at different concentrations level.

Limit of Detection and Limit of Quantitation

For the evaluation of limit of detection and limit of quantification different concentrations of the standard solutions of stigmasterol were applied along with chloroform as blank and determined on the basis of signal to noise ratio. LOD was determined at an S/N of 3:1 and LOQ at an S/N of 10:1.

Specificity

The specificity of the method was ascertained by analyzing standard Stigmasterol and extracts. The spot for Stigmasterol in sample was confirmed by comparing the R_f and spectra of the spot with that of sample. The peak purity of Stigmasterol was assessed by comparing the spectra at three different levels, i.e., peak start, peak middle and peak end positions of the spot/bands.

Robustness

The estimation was performed by varying the selected parameters (mobile phase composition, mobile phase volume and duration of mobile phase saturation) within certain limits ($\pm 10\%$) and there has been no notable alteration found on method performance and in results obtained. The developed method thus, can be considered as robust enough. The results were indicated by the %RSD between the data at each variable condition.

Accuracy

The accuracy of the method was measured by performing recovery experiments at three different levels (50%, 100% and 150% addition of stigmasterol) using the standard addition method. The known amounts of stigmasterol standard (100 ng/spot) were added by spiking. The values of % recovery and average value of % recovery for stigmasterol were calculated, which is given in table 2.

System suitability

System suitability tests are performed to verify whether resolution and repeatability were adequate for the analysis. System suitability was determined by applying

freshly prepared standard solution of stigmasterol, concentration 200ng/spot, six times to the same chromatographic conditions then scanned and densitograms were recorded. The measured peak areas for stigmasterol and their retention factor were noted for each concentration of stigmasterol and values of the mean peak area, the standard deviation (SD) and the relative standard deviation (%RSD) were calculated.

Estimation of Stigmasterol in Herbal Extracts

To determine the content of Stigmasterol in the herbal extract, 1gm of powder of plant parts extracted with 50ml of petroleum ether then concentrated and finally diluted with chloroform. The resulting solution is centrifuged at 3000 rpm for 15 min and the supernatant is analyzed for Stigmasterol content. The filtered solution is applied on the TLC plate followed by development and scanning. The analysis is repeated for six times and the possibility of interference from other components of extract in the analysis is studied.

The spot at $R_f = 0.45$ corresponding to Stigmasterol was observed in the chromatogram of the extracts along with other components. There was no interference from other components present in the chromatogram (Fig 3-6). Total Stigmasterol content was calculated (Table 4).

RESULTS AND DISCUSSION

TLC fingerprint and Co-chromatography

Chromatographic fingerprint analysis has shown to be a rational and feasible approach for the quality assessment and species authentication of traditional medicine. It utilizes chromatographic techniques to construct specific patterns of recognition for herbal drugs. The developed fingerprint pattern of components can then be used to determine not only the absence or presence of markers of interest but the ratio of all detectable analytes as well. Although high performance thin layer chromatography (HPTLC) has a few limitations, such as the limited developing distance and lower plate efficiency by comparison with HPLC and GC, it is still an effective tool for quality evaluation of herbal drugs due to its simplicity, low cost, and requirement, and it has been successfully utilized to develop the chromatographic fingerprint for botanical drugs. Moreover, the above mentioned shortcomings can be overcome by separately developing fractions of different polarity on two or several thin layer plates. Thus, the unique feature of the picture like image of HPTLC coupled with digital scanning profile is gradually attractive to herbal analysis to construct the herbal chromatographic fingerprint. This HPTLC could provide adequate information and parameters for comprehensive identification and differentiation of two closely related herbal medicines.

TLC densitometric quantification of stigmasterol using HPTLC

There is no report of quantification of stigmasterol in leaves, stems and flower of *Ageratum conyzoides* by HPTLC. Hence we developed a simple and precise method for quantification of the marker compound. The TLC procedure was optimized with a view to quantify the herbal extracts. The mobile phase consisting of Chloroform: ethanol (9.8:0.2) gave better, sharp and well-defined peak resolution. The spot at R_f 0.45 was identified as Stigmasterol with the help of chromatogram of the standard compound. The well defined spots were obtained when the chamber was saturated with mobile phase for 20 minutes at room temperature.

The TLC plate was visualized at 360nm and 490nm after derivatization. A photograph of a TLC plate after chromatography of stigmasterol standard and extracts of dried leaf, stem and flower of *Ageratum conyzoides* showed the identity of stigmasterol bands in the sample chromatogram were confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution (Fig. 3) and by comparing retention factor of stigmasterol from sample and standard solution. The peak corresponding to stigmasterol from the sample solution had same retention factor as that from stigmasterol standard (R_f 0.45) (Fig. 4-6).

The TLC densitometric method was validated in terms of precision, repeatability, and accuracy (Tables 1-3; figure 1, 2). The linearity range for stigmasterol were 50-300ng/spot with correlation coefficient, intercept and the slope 0.999, 11.163 and 690.892 respectively (Y = 11.163X + 690.892).

The measurement of the peak area at 5 different concentrations levels showed low values of %CV (< 2%) for inter-day (0.46-1.75) and intra-day (0.44-1.8) variation for different concentrations of stigmasterol which suggested an excellent precision and reproducibility of the method (table 3).

The limits of detection (LOD) and quantification (LOQ) were 15ng and 45ng respectively which indicate the adequate sensitivity of the method (table 1).

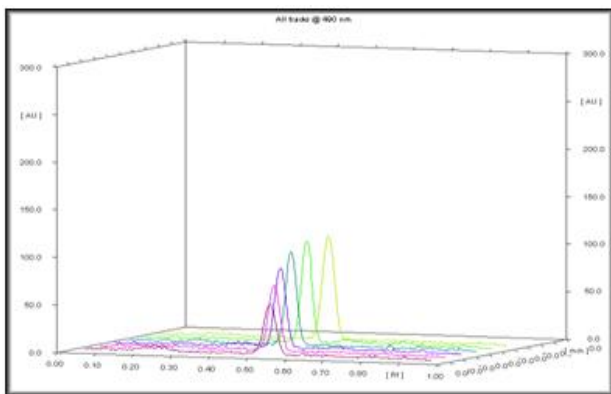


Figure 1: Calibration Curve for Standard Stigmasterol (50-300ng/spot) (3D View).

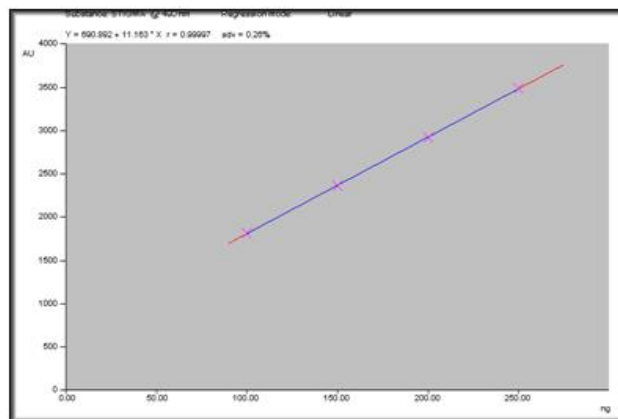


Figure 2: Calibration Curve plot for Stigmasterol.

Table 1: Method Validation Parameters for Quantification of Stigmasterol by proposed TLC densitometric method

Parameters	Method (Stigmasterol)
Selectivity	Selective
Specificity	Specific
Linear range (ng/spot)	50-300
Correlation coefficient (r ²)	0.99997
Linear Regression Equation Y = mX + c	Y = 11.163X + 690.892
LOD (ng/spot)	15
LOQ (ng/spot)	45
% Recovery	98.66
Repeatability (%RSD, n=6)	0.2805%
Precision (%CV)	
Intraday (n=6)	0.44-1.8
Interday (n=6)	0.46-1.75

Table 2: Recovery studies of stigmasterol at 50%, 100% and 150% addition by the proposed TLC densitometric method

Concentration of standards (ng/spot)		Area spotted		Total Area (Sample + Standard)	Total Area Obtained	% Recovery
Sample content	Spiked amount	Sample Area	Spiked area			
100	50	1565	741	2306	2300	99.7
100	100	1565	1558	3123	3107	99.48
100	150	1565	2362	3927	3800	96.77
Mean Recovery						98.66

Results from recovery studies, listed in table 2, were within acceptable limits (98.66%), indicating the accuracy of the method was good. Low % RSD value of 0.28% (between the peak area values) proved the ruggedness of the method indicating that stigmasterol is stable during the extraction procedure as well as during analysis.

The contents of Stigmasterol quantified using TLC densitometric methods were found to be 0.1168±0.05, 0.289±0.02 and 0.0188±0.01 %w/w, respectively in

leaves, stems and flower of *Ageratum conyzoides* (Table 4; figure 4-6).

Table 3: Intra and Inter day Precision of HPTLC method (n=6)

Amount ng/spot	Intra day precision		Inter day Precision	
	SD in Area	%CV	SD in Area	%CV
50	6.78	0.91	13.29	1.75
100	6.93	0.44	14.57	0.93
150	26.85	1.15	30.83	1.32
200	28.2	0.98	27.92	0.98
250	61.98	1.8	15.38	0.46

Table 4: Amount of Stigmasterol found in *Ageratum conyzoides* in % w/w.

<i>Ageratum conyzoides</i>	% of Stigmasterol Mean ± SD
Leaf (A5)	0.1168±0.05
Stem (A6)	0.289±0.02
Flower (A7)	0.0188±0.01

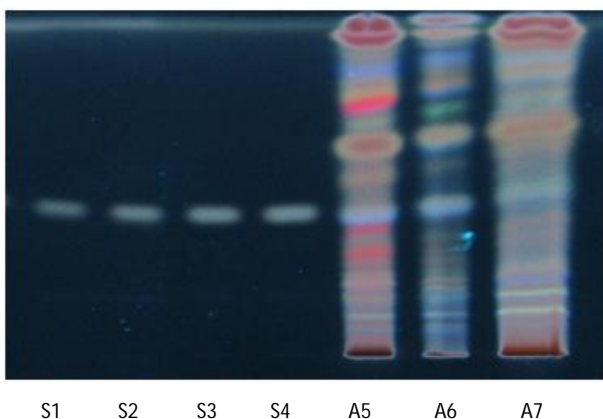


Figure 3: HPTLC chromatogram of Standard Stigmasterol (50-300 ng/spot) and Petroleum Ether extracts of *Ageratum conyzoides* (Leaf A5, Stem A6, Flower A7) using Solvent System:- Chloroform: Ethanol (9.8:0.2), after derivatization with 5% methanolic sulphuric acid and observed under 366nm and under Visible (490nm).

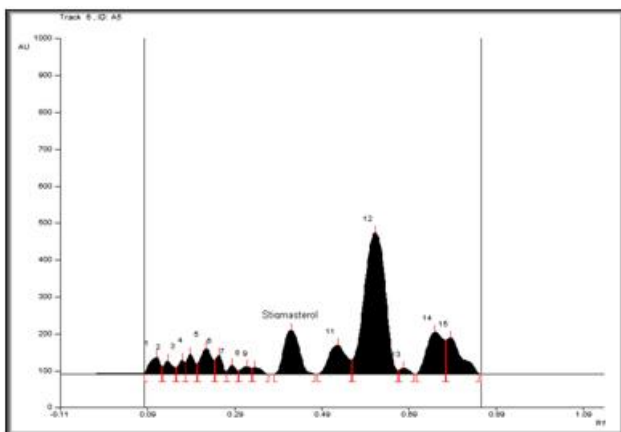


Figure 4: Densitometric chromatogram of Petroleum Ether extracts of Leaf (A5) of *Ageratum conyzoides* at 490nm.

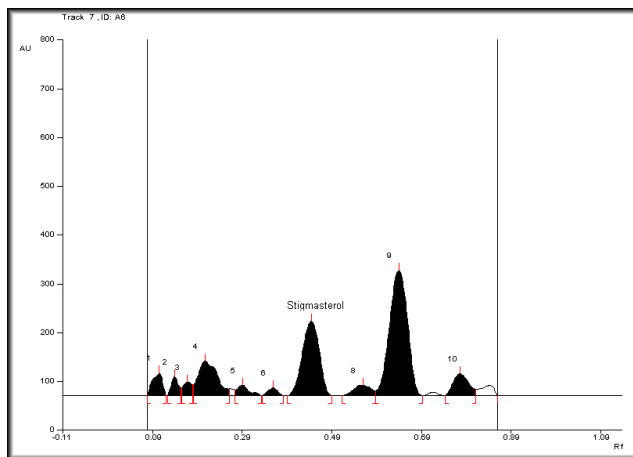


Figure 5: Densitometric chromatogram of Petroleum Ether Extracts of Stem (A6) of *Ageratum conyzoides* at 490nm.

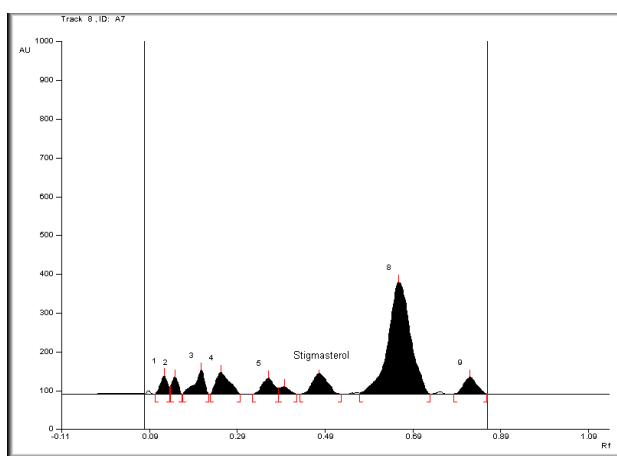


Figure 6: Densitometric chromatogram of Petroleum Ether Extracts of Flower (A7) of *Ageratum conyzoides* at 490nm

CONCLUSION

A rapid, simple, precise, accurate and specific HPTLC method for quantitative estimation of stigmasterol present in the dried leaves and stems of *Ageratum conyzoides* has been developed and validated. The data could be used as a quality control standard. The method used in this work resulted in good peak shape and enabled good resolution of stigmasterol from other constituents of the plant materials. Because recovery (98.66%) was close to 100%, there was no interference with the stigmasterol peak from other constituents present in the plant. The HPTLC images shown in figure 4-6 indicate that all sample constituents were clearly separated without any tailing and diffuseness.

A comparative study of this biomarker compound has shown that it is present in higher amount in the leaves while the concentration was low in the stem extract of *Ageratum conyzoides*. The activity of a plant extract is always influenced by the quantity of active principle present in the extract. Since Stigmasterol is used in the variety of different diseases, it is very essential to develop a standardization method from which one can optimize its quantity in the herbal formulations.

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