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Development and validation of isocratic RP-HPLC method for the quantification of stigmasterol in the crude extract of *Vernonia anthelmintica*

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ABSTRACT

A method for separation and quantification of stigmasterol by reverse-phase high performance liquid chromatography (HPLC) was developed and validated. Stigmasterol was used as calibration standard for the quantification present in the crude methanolic extract of seeds of *Vernonia anthelmintica* extract were analyzed. The analysis was performed using a Thermo Scientific Hypersil C18 column (250 x 4.0 mm i.d., 5 µm particle size), as stationary phase, with a flow rate of 1 ml/min and detection at a wavelength of 210 nm. In this study, an excellent linearity was obtained with r higher than 0.99. With other validation data, including precision, specificity, accuracy and robustness, this method demonstrated good reliability and sensitivity, and can be conveniently used for the quantification of stigmasterol in crude methanolic extract of seeds of *Vernonia anthelmintica*.

Keywords: *Vernonia anthelmintica*, HPLC, stigmasterol, validation

INTRODUCTION

Vernonia anthelmintica, belongs to the family compositae commonly known as kalijiri, is an important medicinal plant of India. It has a good anthelmintic property and used for the treatment of various skin infections. It is also reported to be used in asthma, kidney troubles, cough, and diabetes [1, 2]

Research has indicated that stigmasterol may be useful in prevention of certain cancers, including ovarian, prostate, breast, and colon cancers [3]. Studies have also indicated that a diet high in phytoesterols may inhibit the absorption of cholesterol and lower serum cholesterol levels by competing for intestinal absorption. Studies with laboratory animals fed stigmasterol found that both cholesterol and sitosterol absorption decreased 23% and 30%, respectively, over a 6-week period. It also possesses

potent antioxidant, hypoglycemic and thyroid inhibiti ng properties. [4]

Chemical and chromatographic techniques may be used to aid in the identification of an herbal material or extract. Chromatographic techniques such as HPLC, TLC, GC and capillary electrophoresis and spectroscopic methods such as IR, NMR and UV may also be used for fingerprinting. In order to control the quality of herbal drugs in a better way, we must develop new techniques and terms to the maximum extent. The development and validation of an efficient analytical method is an integral part of

the quality control of the source material, to guarantee the safety and effectiveness of the resulting compound.[5,6] Thus, considering the pharmacological potential of *Vernonia anthelmintica* and the lack of specifications for the quality control of this plant raw material, a validated HPLC method was developed

The objective of the present study was to develop and validate a method for the quantitative analysis of stigmasterol by HPLC obtained from the methanolic extract of the seeds of *Vernonia anthelmintica*. This will provide the scientific basis for the quality control of extracts prepared from this plant. The following validation characteristics were assessed: specificity, linearity, limit of detection and quantification, accuracy, precision and robustness.

MATERIALS AND METHODS

Seeds of *Vernonia anthelmintica*, compositae were collected from the local market of Coimbatore. The plant material was identified by Dr.C.Kunhikannan, Scientist F and Head of office, Institute of forest genetics and tree breeding, Coimbatore, bearing the Ref.No.940/BD/ID/IFGTB/2017

Preparation of the extract

The seeds of *Vernonia anthelmintica* were powdered coarsely. The coarse powder was refluxed to hot continuous extraction with different solvents such as petroleum ether, chloroform, ethyl acetate and Methanol in the increasing order of polarity using soxhlet apparatus .Extraction was continued until all the constituents are extracted. All the extracts were concentrated and evaporated to dryness. The dried extracts were subjected to preliminary phytochemical analysis and quantification.

Chemicals and reagents

All reagents and solvents were analytical and HPLC. Stigmasterol (Sigmaaldrich) of the highest grade (purity>98.0%) was used as the external standards.

Instrumentation and chromatographic conditions

The analyses were carried out using an HPLC system (Shimadzu, SPD-M10AVP) consisting of a solvent delivery pump (Model LC-10 ADvp), a variable wavelength PDA detector (Model SPD 10 AVP), a manual injection valve (Rheodyne®, USA) with a 20 µL loop, and degasser (DGU 14A). Data collection and analyses were performed using LC-MS solutions Software. A gradient elution was performed on a Thermo Scientific Hypersil C18 column (250 x 4.0 mm i.d., 5 µm particle size) (Thermo Scientific, USA). The mobile phase consisted of acetonitrile: methanol: buffer (0.1% orthophosphoric acid) in the ratio 70:25:5. All solutions were degassed and filtered through a 0.45 µm pore size filter (Millipore, USA). Separations were effected by an isocratic elution. The mobile phase flow rate was 1 mL/min and the injection volume was 20 µL. UV detection was performed at 210 nm. Using these chromatographic conditions, it was possible to confirm the retention time of stigmasterol.

Preparation of standard solution

An accurately weighed appropriate amount of the reference compound stigmasterol was dissolved in methanol in a 100-ml volumetric flask, to obtain a stock solution. The concentration of the compound in this solution was 1000 µg/ml. Working solutions were prepared by stepwise dilution of the stock solution with methanol for concentrations ranging from 20-300µg/ml.

Method validation

In the validation of the analytical method used for the quantification of stigmasterol in the methanolic extract of *Vernonia anthelmintica*, the following parameters were determined as per ICH guidelines. [7]

Specificity

Specificity is the ability of a method to discriminate between the study analyte(s) and other components in the sample. Specificity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients. The blank mobile phase, placebo and assay sample were injected in the chromatographic system. The chromatogram obtained was evaluated for any additional peaks and changes in the retention time of the analyte. The spectrum of the sample solution was compared with that of standard. The peak purity was assessed from peak purity index values.

Linearity

The linearity between peak area and concentration was analyzed using calibration curve obtained with standard solutions at five different concentrations of

the standard. The concentration levels used to construct calibration curves were from 20-300µg/ml. The data for peak area versus drug concentration were treated by linear regression analysis.

Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curves of the standard. LOD was calculated according to the expression 3.3xSD/S where SD is the standard deviation of the response and S is the slope of the calibration curve. LOQ was established by using the expression 10 x SD/S

Accuracy

The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the standard to the sample, at three different levels (50%, 100% and 150%) of the initial concentration of the sample. For evaluation of recovery of stigmasterol standard was added to the sample whose final concentration were 150, 200, 250 µg/ml of this compound. Average recoveries were calibrated by the formula recovery (%) = {(amount found - original amount)/amount spiked} x 100.

Precision

The precision of the method was investigated with respect to repeatability, intermediate precision (inter-day variation) and reproducibility by determination of standard solution at 100% of the test concentration. To assess the intra-day precision (repeatability) of the method, the sample was injected six times within a day. The inter-day precision was determined with the sample assayed on different days and by another analyst. Reproducibility was determined by injection of the sample six times and consequently comparing the results in two different laboratories. Three sample solutions were prepared and analyzed under the conditions established and by changing the wavelength parameter from 190 to 210.

Robustness

The responses of deliberate variations in chromatographic conditions such as changes in flow rate by ± 0.1ml/min, volume of organic solvent in mobile phase by ± 0.5% have not affected the chromatogram and hence the developed method is said to be robust.

Quantification of stigmasterol from ethanolic extract of *Vernonia anthelmintica*.

The optimized HPLC method was used to estimate stigmasterol in the extract of *Vernonia anthelmintica*. For the present study, 30 mg of crude extract were dissolved in 10 ml methanol. The sample of this concentration was used for the analysis and volume of 20µl was injected and responses were recorded. Further all the prepared extracts were injected and the chromatograms were recorded (Figure 2-4)

RESULTS AND DISCUSSION

The HPLC method carried out in this study was aimed at developing a chromatographic system, capable of eluting stigmasterol in the crude methanolic extract of *Vernonia anthelmintica*. In the development of the HPLC method for determination of stigmasterol in the methanolic extract, several solvent systems (methanol-water, acetonitrile-water, and Phosphoric acid-buffer) were evaluated and compared. The choice of detection wavelength was determined by performing a screening with 10 ppm of stigmasterol in methanol in a spectrophotometer UV/ VIS. The UV spectra were recorded from 190 to 210 nm and exhibited maximum wavelengths at 210 nm. The optimization of the chromatographic conditions led to a good peak shape when compared to other methods previously described for the quantification of this compound. The result for quantification of the stigmasterol in the extract of *Vernonia anthelmintica* was 29.43µg/mg. It is important to note that this is the first report of quantification of sterol in crude extracts of Vernonia anthelmintica. The specificity of the method was evaluated by analysis of the blank, standard and sample solution chromatograms In relation to asymmetry, the peaks showed values of 1.069 for stigmasterol (Figure 2). Linearity was evaluated by the correlation coefficient r, and all values for the compounds were greater than 0.995, showing that responses for the standard in the concentration ranges examined were linear. The results display a coefficient of variation as per ICH guidelines as shown in table 1. The results obtained for the system precision are within the prescribed limits. (Table 3, 4) The results also indicated the minimum concentration levels at which the analyte can be reliably detected and quantified. Recovery of each substance was obtained from the calculated amount found and

original amount (Table 2). Also, there were no significant differences between assay results, indicating that the precision of the proposed method was satisfactory.

Table 1. Linearity data for stigmasterol

S. No	Concentration (µg/ml)	Peak area
1	20	56610
2	50	144687
3	100	247946
4	200	439265
5	300	656348

Table 2. Recovery data of stigmasterol

Conc. of drug taken (µg/ml)	Conc. of standard added (µg/ml)	Mean Conc. found ± SD*	Mean Recovery (%)	% RSD*
100	50(50%)	149.63±0.2809	99.75	0.1877
100	100 (100%)	199.98±0.1717	99.99	0.0860
100	150(150%)	249.73±0.1568	99.89	0.0627

*n=6

Table 3. Repeatability data of stigmasterol

Concentration	Peak area	%RSD
100 µg/ml	247948	0.0014
	247946	
	247940	
	247941	
	247939	
	247938	

Table 4. Intra-day and inter-day precision data of stigmasterol

Conc. (µg/ml)	Intra-day precision		Inter-day precision	
	Mean Conc. ± SD*	% RSD*	Mean Conc. ± SD*	% RSD*
50	49.66±0.2567	0.5169	49.66±0.2382	0.4796
100	99.70 ±0.1642	0.1647	99.71±0.147	0.1475
200	199.57±0.2043	0.1023	199.58±0.2045	0.1024

Table 5. System suitability studies for estimation of stigmasterol by HPLC

S. No	Parameters	Stigmasterol
1.	**Linearity range**	**20-300µg/ml**
2.	**Correlation coefficient**	**0.997**
3.	**Asymmetric factor**	**1.069**
4.	**Theoretical plates**	**1358**
5.	**LOD**	**3 µg/ml**
6.	**LOQ**	**10 µg/ml**
7.	**Retention time**	**9.8mts**

Table 6. Analysis result of extract by developed HPLC method

Extract	component	Amount foundµg/mg
Vernonia anthelmintica	stigmasterol	29.43µg/mg

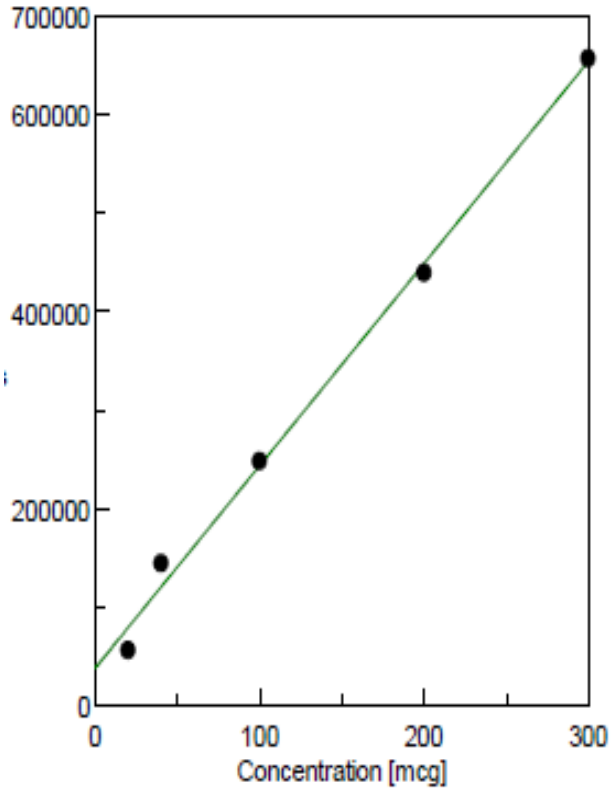

Fig 1. Linearity graph for stigmasterol

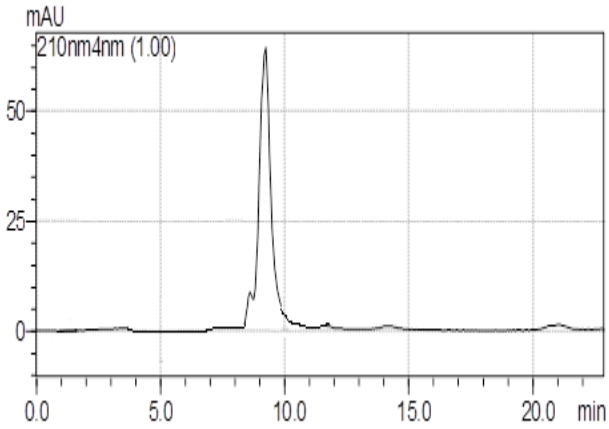

Fig 2. HPLC chromatogram of standard stigmasterol

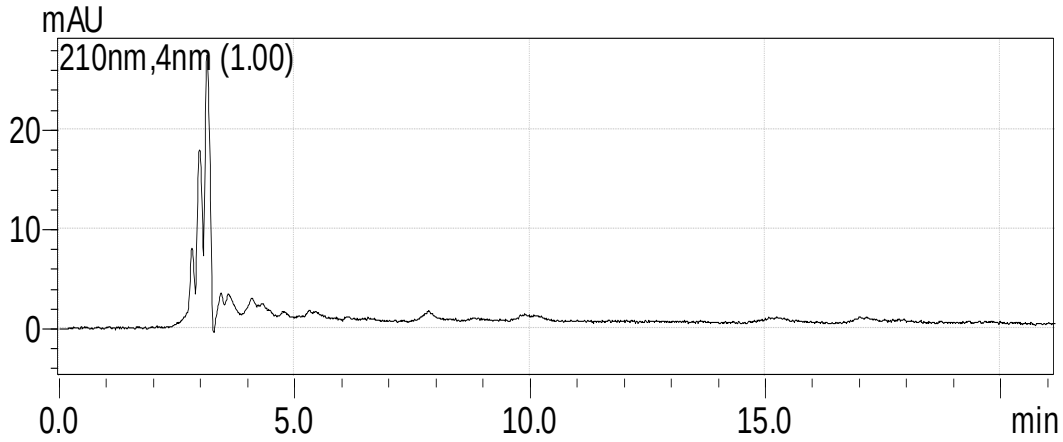

Fig 3 HPLC chromatogram of chloroform extract of *Vernonia anthelmintica*

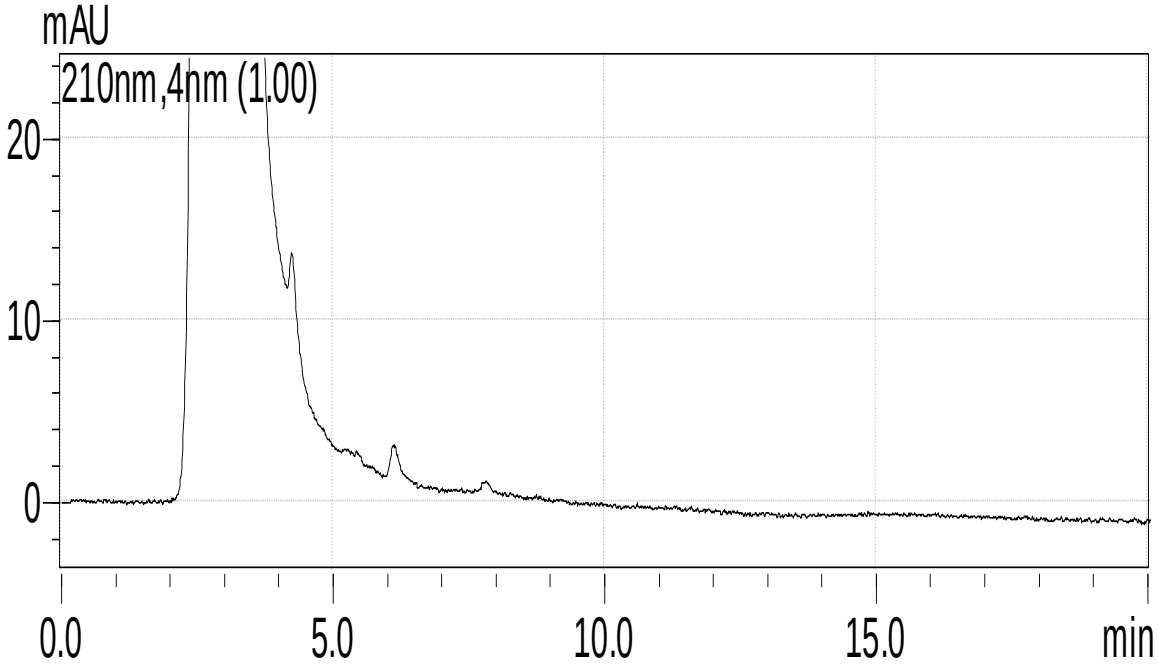

Fig 4. HPLC chromatogram of ethyl acetate extract of *Vernonia anthelmintica*

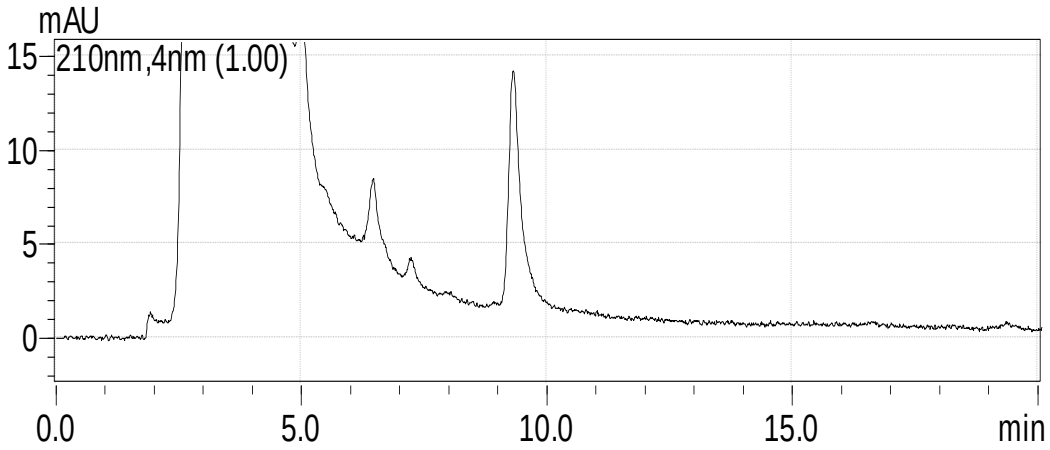

Fig 5. HPLC chromatogram of alcoholic extract of *Vernonia anthelmintica*

CONCLUSION

A simple, specific, precise, rapid and reproducible HPTLC method has been developed for the quantification of stigmasterol in the extract of *Vernonia anthelmintica*. This method showed good linearity relationship between the peak area and concentrations which is acceptable and reproducible. The method can also be adopted to quantify stigmasterol in other plant species. This validation procedure confirms that this an appropriate method for the quality control of *Vernonia anthelmintica* extract.

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