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IDENTIFICATION AND QUANTIFICATION OF BETA-SITOSTEROL IN *LEUCAS ASPERA* LINN. BY HPTLC-MS

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ABSTRACT

Leucas aspera linn. is commonly called as 'Pandharpheda' and is widely distributed throughout south India, having many medicinal properties. A sensitive, simple and accurate HPTLC method has been established for identification and quantification of beta-sitosterol from *leucas aspera* linn. whole plant powder. Quantification was carried out on pre-coated plate of HPTLC silica gel 60 F_{254} using mobile phase (toluene: ethyl acetate: formic acid, 12:7:1,v/v/v). CAMAG scanner IV set at visible light (520nm) for detection and quantification. The percentage concentrations of the beta-sitosterol were found to be 0.0672.Response was linear over the range of 0.1µg to 0.5µg with 0.66 and 0.314 of %CV respectively.CAMAG TLC-MS interface was used for the confirmation of m/z values of beta-sitosterol in standard as well as sample. The m/z value was found to be 397($M+H^+-H_2O$). The method i.e. HPTLC and TLC-MS together provides a rapid and secure method for detection, identification and quantification of biomarker beta-sitosterol.

KEY WORD: Leucas aspera linn., CAMAG HPTLC, CAMAG TLC-MS interface, Shimadzu LCMS-2020, Beta-sitosterol, Identification, Quantification.

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INTRODUCTION

Leucas aspera linn. belongs to Lamiaceae (mint family) . It was found in southern parts of India and planted in gardens, temples and it is commonly called as 'Pandherpheda'[1].Ethnomedicinally, it is used in anti-inflammatory[2], antibacterial[3], antioxidant and prostaglandin[4]. The whole plant of *leucas aspera* linn., reportedly contains beta-sitosterol[5-6], eugenol, citral, linalool, thymol[5], L-arginine, L-cysteine, glycine[6], palmitic, steric, oleic, linoleic acids[7] as chemical moieties. Beta-sitosterol is a secondary metabolite, which is a bio-active compounds[8].Beta-sitosterol is reported to help in curing cholesterol absorption, immune-modulator, breast cancer and gynecological disorders [9,10]. HPTLC is an important tool that can be used for qualitative and quantitative analysis for checking the purity and identity of botanical materials and also for quality control of finished product [11,13,14,15]. Literature revealed that there is no HPTLC method available for quantification of beta-sitosterol from whole plant powder of *leucas aspera* linn. HPTLC method was developed for the separation of beta-sitosterol from the whole plant powder of *leucas aspera* linn. Beta-sitosterol was quantified by using densitometric technique and also identified from the same HPLTC plate by coupling of HPTLC with mass spectrometry via the TLC-MS interface. Time needed for mass spectrometric detection in HPTLC-MS is very low compared to HPLC-MS because mass spectra can be recorded only of zones of interest [12].

MATERIALS AND METHODS

1) Collection and authentication of plants

Whole plant material was collected from Kollam district, Kerala and herbarium of *leucas aspera* linn. was prepared and authenticated from Dept. of the botany MS University, Vadodara India. The plants collected were washed under running tap water. The plant kept for drying in oven at temperature 40±2°Cfor 10 to 11days. The dried plant material was used for further studies.

2) Standard collection

Standard beta-sitosterol (98.0% purity) was procured from Sigma Aldrich Chemie (Steinheim Germany).

3) Chromatographic conditions

i) Standard preparation

Weighed 10mg of standard beta-sitosterol and dissolved in 10ml of methanol. (conc.: 1.0mg/ml) 1ml of above standard solution is diluted to 10ml with methanol (conc.: 0.1mg/ml).

ii) Sample preparation

Weighed 500 mg of fine powder of *leucas aspera* linn. suspended in 5.0ml of methanol, sonicated for 30mins. Filter through Whatman no 41 (conc. of extract : 100.0mg/ml).

iii) Instrument

CAMAG sample applicator i.e. Linomat-V was used for sample application, twin trough chamber was used for the development and for documentation, TLC-Visualizer was used. Densitometer i.e. Scanner IV was used for scanning the HPTLC. Win CATS system manager s/w was used to control and link all instruments. CAMAG TLC-MS interface was used to elute fractions from plate into MS. LCMS-2020 system (single quadruple mass spectrometer from Shimadzu, Japan) was used for the molecular mass confirmation.

iv) HPTLC plate

Merck pre-coated aluminum HPTLC silica gel $60F_{254}$, Plate size: 20cm X 10cm thickness of absorbent: 0.20mm.

v) Mobile phase

Toluene: ethyl acetate: formic acid, (12:7:1,v/v/v)

Vi) Detivatization

After development plate was dried with cold air dryer. The plate was dipped into the dipping chamber contains Anisaldehyde sulphuric acid reagent. The plate was then dried and heated on CAMAG plate heater on 110°C for 5.0 min.
(Note: Rf. Of Beta-sitosterol does not show UV as well as fluorescence. It is only detectable

after derivatization but for detection on LC-MS system, derivatization is not required.)

vii) Scanning wavelength (after derivatization)

At 520nm using W lamp at absorbance mode in white light

Viii) TLC-MS condition

A non- derivatized chromatogram was used. TLC-MS interface (oval elution head 4X2 cm) coupled with LCMS-2020 (Shimadzu) system with N_2 gas pressure 4 to 6 bars. A: 0.1% formic acid in water, B: acetonitrile (A: B, 20:80)V/V was eluent, pumped at the rate of 0.3ml/min.

ix) Mass conditions

1) MS parameters

i) Desolvation line temperature: 250°C
ii) Nebulizing gas flow: 1.5 L/min
iii) Heat block temperature :350°C
iv) Drying gas flow: 10L/min.
v) Analysis mode: ESI(Electro Spray Ionization)+ve

2) LCMS model: LCMS-2020 (Shimadzu)

RESULTS

Figure.1
3-D plot of densitometric scans of the standard samples at 520 nm (visible light)

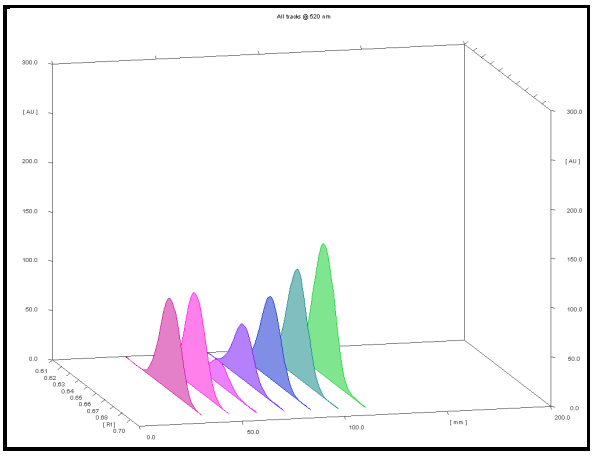


Figure.2
Image of HPTLC plate in visible light after derivatization with anisaldehyde sulphuric acid

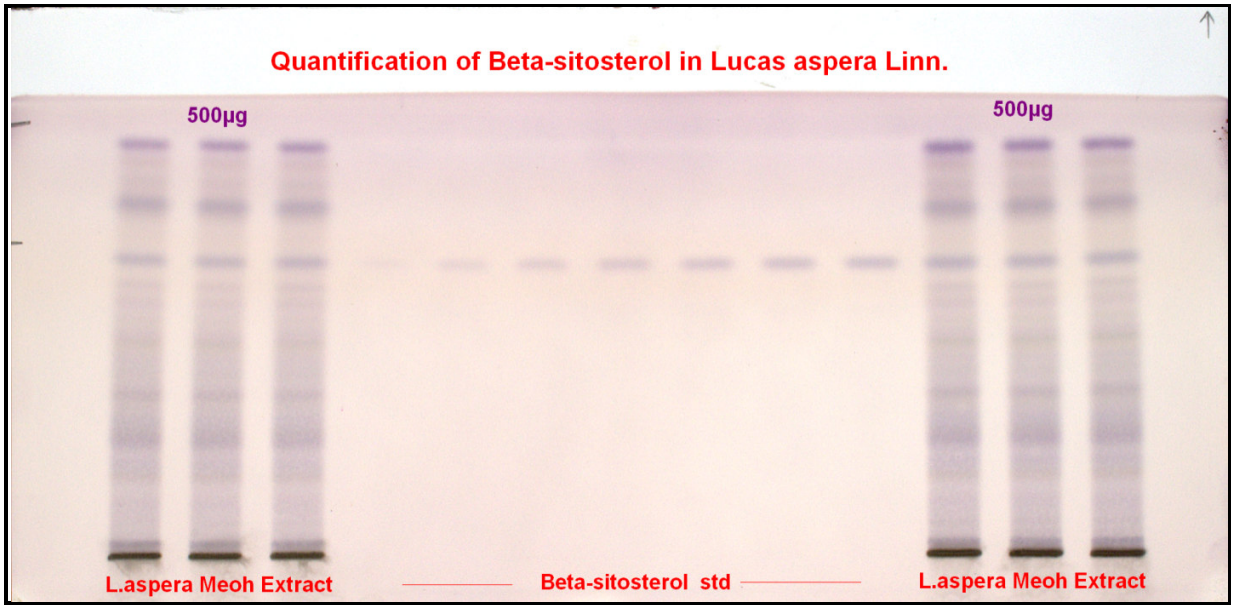
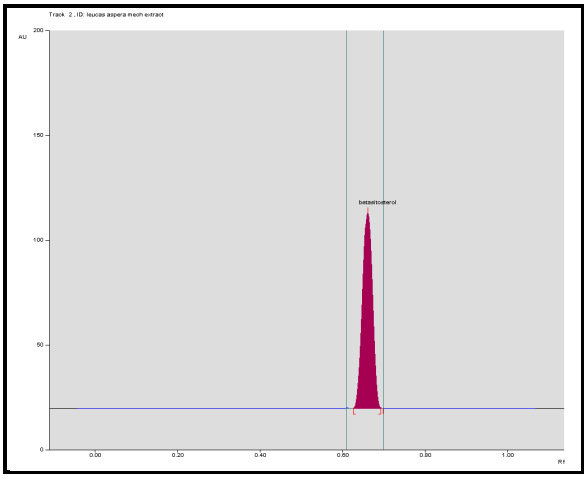


Figure.3
Scan of track 2 in Leucas aspera linn. at 520 nm

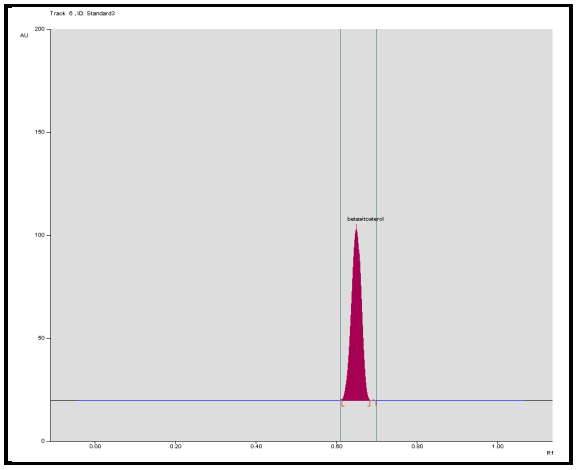


Quantitative data

Track 2, ID: leucas aspera meoh extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.63 Rf	0.1 AU	0.66 Rf	93.4 AU	100.00 %	0.69 Rf	0.4 AU	1884.8 AU	100.00 %	betasitosterol

Figure.4
Scan of track6 (standard beta-sitosterol) at 520 nm



Quantitative data

Track 6, ID: Standard3

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.62 Rf	0.5 AU	0.65 Rf	83.3 AU	100.00 %	0.68 Rf	0.2 AU	1686.2 AU	100.00 %	betasitosterol

Figure 5
calibration graph at 520 nm (0.1µg - 0.5µg) of beta-sitosterol.

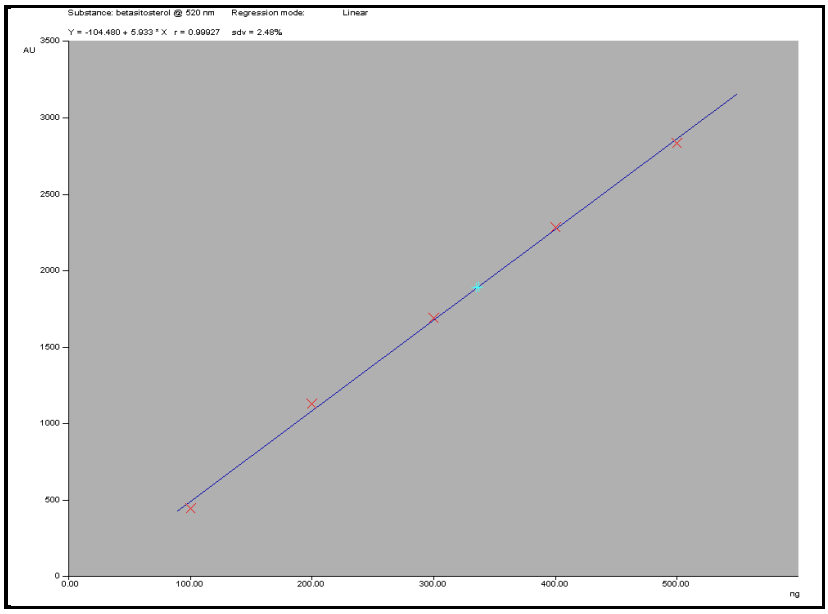


Table.1
summary of beta-sitosterol analysis

Substance: betasitosterol @ 520 nm Regression mode: Linear

Regression via area Y = -104.4803 + 5.9333 * X r = 0.99927 sdv = 2.48 %

Track	Vial	Rf	Amount Fraction	Height	X(calc)	Area	X(calc)	Remark
1	2							Sample leucas aspera meoh extract not evaluated
2	2	0.66		93.44	0.0 ng	1884.77	335.27 ng	Sample leucas aspera meoh extract
3	2	0.66		95.84	0.0 ng	1893.64	336.76 ng	Sample leucas aspera meoh extract
4	1	0.66	100.00 ng	23.56		445.19		Std Level 1
5	1	0.65	200.00 ng	56.22		1128.16		Std Level 2
6	1	0.65	300.00 ng	83.25		1686.19		Std Level 3
7	1	0.65	400.00 ng	109.19		2284.14		Std Level 4
8	1	0.65	500.00 ng	132.98		2833.83		Std Level 5
9	1							Std Level 6 not evaluated
10	1							Std Level 7 not evaluated

Table.2
summary of calibration results of beta-sitosterol analysis

Summary of calibration results per analysis

Sample from vial 2: leucas aspera meoh extract

Result via area

Substance	Rf	X(Average)	CV [%]	n	Remark
betasitosterol	0.66	336.02 ng	0.314	2	

Figure.6
Mass chromatogram of standard beta-sitosterol and sample

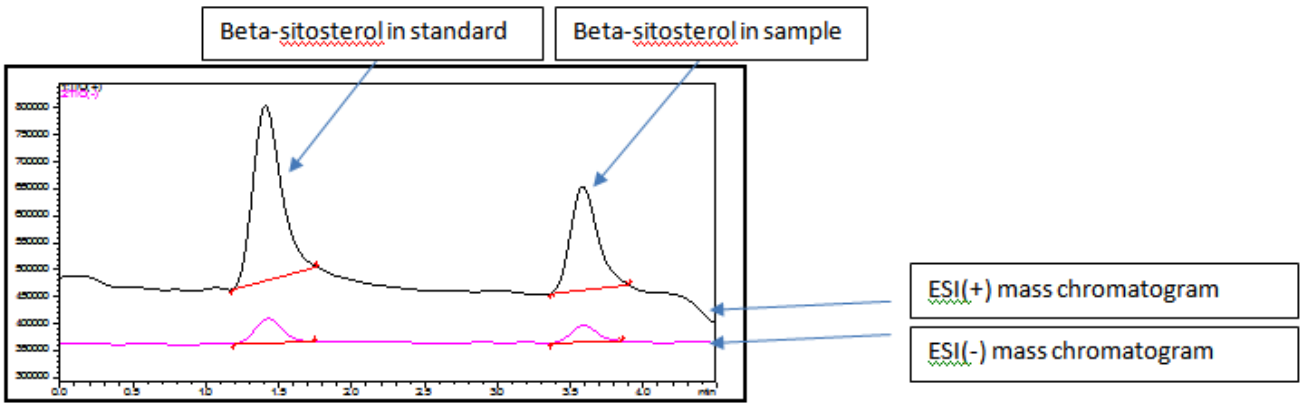


Figure.7
Mass spectra of standard

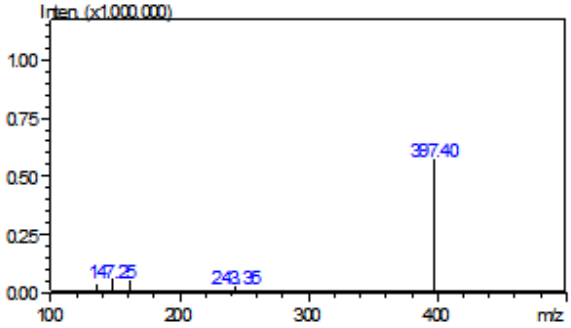
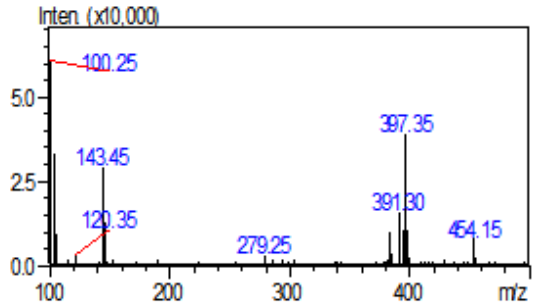


Figure.8
Mass spectra of sample



DISCUSSION

The chromatographic conditions described above, the Rf value of beta-sitosterol was about 0.66 in whole plant of *leucas aspera* linn. extract. The respective Rfs obtained for each track was shown in the table no 1 .The chromatogram of standard beta-sitosterol is shown in figure no 4 and that of the beta-sitosterol in *leucas aspera* linn. is shown in figure 3. The 3D plots of all tracks scanned at 520nm at visible light are shown in figure 1. The area under the curve (AUC) obtained for various tracks are enumerated in table 1. The calibration curve was linear in the range of 0.1µg to 0.5µg, as illustrated in table no 1 and shown in figure 5. The % CV values were found to be less than 2%, indicating that the selected method is precise and reproducible. The estimated value on per gram basis of drug was about 0.0672mg/gm in whole plant. The chromatogram of the mass spectra of standard beta-sitosterol and sample shown in the figure no:6. Mass spectra of standard beta-sitosterol and sample are shown in the figures 7&8. Beta-sitosterol has no absorbance in UV nor does fluorescence hence do derivatization with ASR become necessary it is easy method to quantify the bio marker in the *leucas aspera* linn through HPTLC. Actual molecular weight of the beta-sitosterol is 414.7 g/mol but during the ionization water molecule was eliminated and protonation took place hence mass spectra was observed at 397 m/z value ($M+H^+-H_2O$).

CONCLUSION

From the above method, it shows that a quick and easy approach for the identification and quantification of bio-active beta-sitosterol in *leucas aspera* linn. by HPTLC Detection of beta-sitosterol by TLC-MS for confirmation was done because mass is the best tool for identification. Thus, beta-sitosterol can be used as phytochemical marker for quality control (QC) test of the raw materials. The authors further aim to validate the method in terms of accuracy, reproducibility and recovery. This paper has shown the straight-forward and robust operation of the HPTLC-MS interface which opens a new possibilities into analysis of pharmaceuticals as well as in the analysis of natural products directly from HPTLC plate[13-15].

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