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PHYTOCHEMICAL SCREENING, DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR ISOLATED STIGMASTEROL FROM *OCIMUM SANCTUM*.

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ABSTRACT

Stigmasterol is the abundant phytoconstituent present in the leaves of *Ocimum sanctum*, having a major function to maintain the structure and physiology of cell membranes. The dried leaves of *Ocimum sanctum* was subjected to extraction, preliminary phytochemical screening and then isolation. The identification of the isolated compound was confirmed by TLC, HPLC profile and spectral studies by comparing with the standard. RP-HPLC method were developed and validated as per ICH guidelines using the isolated biomarker compounds such as stigmasterol. Results of HPLC analysis of isolated compound shows the presence of stigmasterol which is confirmed by the single peaks present in the chromatogram at 3.18 retention time (in mins). The reported data may be of great value as a reference standard for evaluation of the isolated compound.

KEYWORDS: *Ocimum sanctum*, stigmasterol, RP-HPLC.

INTRODUCTION

The interest for herbs are developing step by step all through the world and major pharmaceutical organizations are at present board research take a shot at plant materials for their potential restorative value.[1] The tranquilize revelation process is developing costly step by step. The drug discovery process is more complex and risky investment, drugs in the development pipeline are failing at the end of Phase 3 or Phase 4, making it more expensive and time consuming. Powerful new technologies such as high throughput systems and combinatorial chemistry are revolutionizing drug discovery, but natural products still offer unmatched structural varieties.[2]

Ocimum sanctum is a traditional or holy Indian herb belonging to family Liliaceae. Traditionally, *Ocimum sanctum* (Tulsi) is taken in many forms like as herbal tea, dried powder, fresh leaf, or mixed with Honey or Ghee. It has endless miraculous medicinal values and diverse pharmacological actions on cardiovascular system, respiratory system, lung infections, etc. Antimicrobial activity of *Ocimum sanctum* has been tested against a wide range of microorganisms.[3] Recent studies show that it is valuable in growth inhibition of HIV.[4] and tumor cells.[5,6]

Plant sterols (i.e. phytosterols) which are cholesterol like substances represent an important group of compounds in unsaponifiable plant oils that confer biological activities to the oils. The rationale behind choosing phytosterols is that they have various medicinal advantages like they have reported antidiabetic activity, anti-inflammatory, anti-fungal, anti-ulcerative, antibacterial and anticancer activities. Therefore, phytosterol enriched products have been engineered and marketed as they lowers serum cholesterol level and thus preventing cardiovascular risk.[7] Stigmasterol (Fig1) is reported to have antioxidant, thyroid inhibitory, anti peroxidative hypoglycaemic.[8,9] hypocholesteromic.[10] and anti-inflammatory activity.[11]

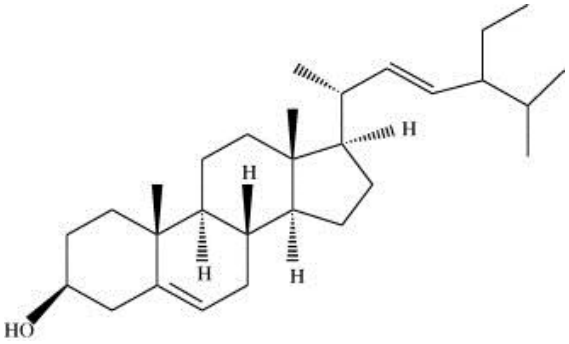

Fig 1: Stigmasterol.

To date, much research has been attentive on the analysis of saponins, but only few studies have focused on phytosterols in *Ocimum sanctum*. Thus, we aimed to analyze the phytosterol content in the leaves of *Ocimum sanctum* using high-performance liquid chromatography (HPLC).

MATERIAL AND METHODS

Plant Material: The fresh leaves of *Ocimum sanctum* (Linn.) were collected from village Jahangir (31˚30'070"N 75˚02'16.0"E) district-Tarn-taran, Punjab, India, in the month of August.

Authentication Of Plant Material: The samples of *Ocimum sanctum* Lin. has been authenticated by **Dr. Parveen Kumar Ahuja**, Assistant Professor, Department of Agriculture, Crop Protection Division (Agriculture Botany), Khalsa College, Amritsar, Punjab, India with Reference number TU-1603181534.

Extraction: Extensive literature survey on the extraction of phytosterols was done and all the methods were tried one by one individually or in combination. Finally, the maximum yield was found with extraction by the process of maceration with ethanol. Coarsely powdered leaves were placed in conical flask and defatted using petroleum ether. The extraction was made by 99% ethanol by maceration process.

Phytochemical Screening: Chemical tests were performed for phytochemical screening of secondary metabolites such as alkaloids, anthraquinone glycosides, cardiac glycosides,[12,13] saponins, glycosides and phytosterols, carbohydrates, phenolic compounds, tannins, flavonoids,[13,14] proteins and amino acids.[15]

Thin Layer Chromatography: Thin Layer Chromatography (TLC) pre-coated plates (Merck: 20x20 cm; with thickness 0.2-0.3mm) were used. The ethanolic extract of Ocimum sanctum was subjected to thin layer chromatography using mobile phase Toluene: Ethyl acetate: Formic acid (8.5:1:0.5). The Rf value of standard and test compounds were compared.[16,17]

Isolation and Fractionation: The column chromatographic assembly (column length x diameter = 400 x 50 mm) was set for the isolation of compounds from extracts. The pre-activated silica gel (60-120 mesh size) was used for column packing. 20g of semisolid extract was accessed into the packed column. The mobile phase in combination (Toluene: Ethyl acetate: formic acid:: 8.5:1:0.5) was used. The solvent level was maintained 2.5 cm above the extract. The isolated fractions were collected drop by drop in separate volumetric flasks.[18]

Characterization

Thin layer chromatography (TLC): The isolated fractions were subjected to thin layer chromatography for the identification of the eluted constituents and then observed under UV at 254 and 365nm.[16]

Fourier-transform infrared (FTIR) spectroscopy: The FTIR spectra of marker compound and isolated stigmasterol were recorded using Shimadzu infra-red spectrophotometer in the wavelength region of 4000 to 400 cm^{-1}.Drug sample and potassium bromide were mixed uniformly and filled into the die cavity of sample holder and an IR spectrum was recorded.[18]

Method Development

Ultra-Violet (UV) Spectroscopy

Determination of λmax: Stigmasterol was uniformly mixed with chloroform and scanned between 200-400nm and the λmax of stigmasterol was found to be 210nm.

Construction of calibration curve: The calibration curve of stigmasterol was made by plotting the absorbance of concentration range of 5, 10, 15, 20, 25µg/ml of solution with chloroform at a λ_{max} of 210nm.

RP-HPLC Method Development

Table 1: Optimized chromatographic conditions for estimation of stigmasterol.

Mobile phase	**Acetonitrile 0.1% acetic acid :: 90:10 v/v**
Pump mode	Isocratic
Diluents	Mobile phase
Column	C-18 column (250×4.6×0.5)
Column temperature	Ambient
Wavelength	210nm
Injection volume	20µl
Flow rate	1.2ml/min
Run time	15 min.
Retention time	3.35 min.

The separation was performed on an Agilent C_{18} column (particle size 5 µm, 250 mm x 4.6 mm) (Agilent technologies, USA). Chromatographic data was recorded and processed using a LC100 Chromatographic Station. Mobile phase consisting of Acetonitrile: 0.1% Acetic acid (90:10) was used. Solvents used were of HPLC grade. Analysis was isocratic at 1ml/min flow rate and sample volume was 20µl. Peaks were integrated at the wavelength of 210 nm.[19]

Preparation of standard stigmasterol solution: Standard stock solution of Stigmasterol 1000 µg/ml was prepared from which working stock solution of 20µg/ml was made by dilution with chloroform.

Preparation of sample stigmasterol solution: Working sample solutions were prepared from 1µg/ml stock solution by diluting with chloroform to furnish five different concentrations of 10, 20, 30, 40 and 50 µg/ml.

VALIDATION: Validation is done as per ICH guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, robustness, system suitability, limit of detection, limit of quantification and solution stability.[20]

RESULTS AND DISCUSSION

Phytochemical screening

Table 2: Phytochemical screening of stigmasterol.

Tests	Observation	Results	
		HLE	ELE
Test for steroid			
a. Salkowski reaction	Chloroform layer appear red and acid layer shows greenish yellow fluorescence	++	+
b. Liebermann – burchard reaction	First red, then blue and finally green colour appears	++	+

Thin layer chromatography

TLC of ethanolic extract of stigmasterol

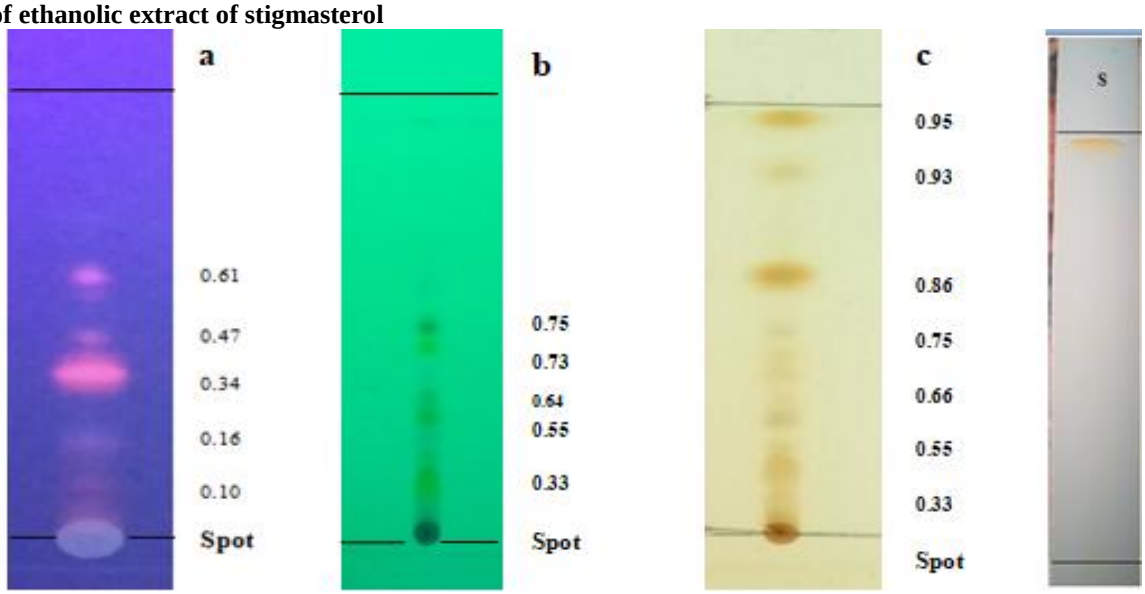

Fig. 2: TLC of ethanolic extract of *Ocimum sanctum* (a) Short wave (b) Long wave (c) Iodine vapour (d) TLC of standard (stigmasterol) and test extract.

Table 3: TLC profile of ethanolic extract of *Ocimum sanctum* leaves.

S. No.	Mobile phase	Rf value		
		Short wave	Long wave	Iodine vapour
1	Toluene: Ethyl acetate: Formic acid (8.5:1:0.5)	0.10, 0.16, 0.34, 0.47, 0.61	0.33, 0.55, 0.64, 0.73, 0.75	0.33, 0.55, 0.66, 0.75, 0.86

TLC of Isolated Stigmasterol

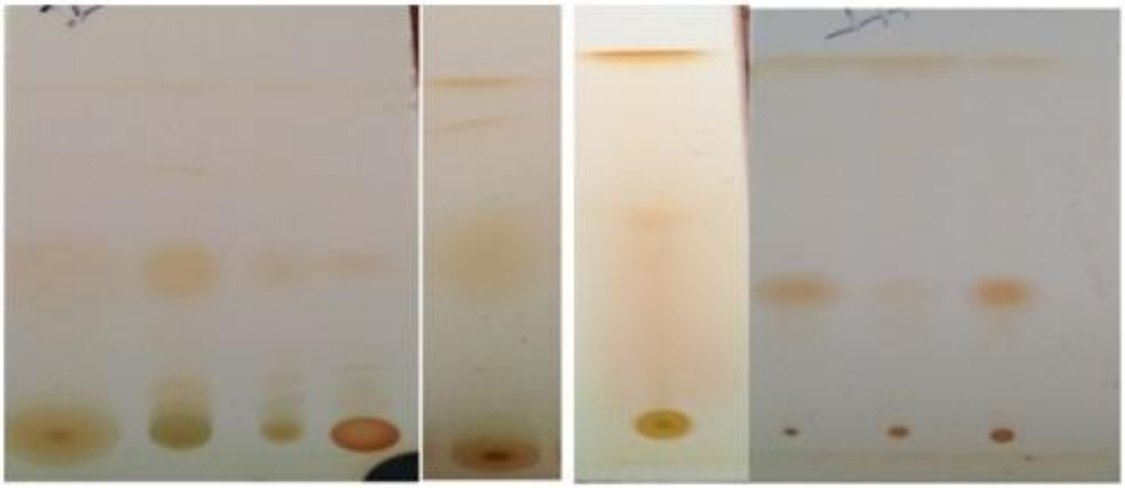

Fig. 3: TLC of isolated stigmasterol.

FTIR spectroscopy

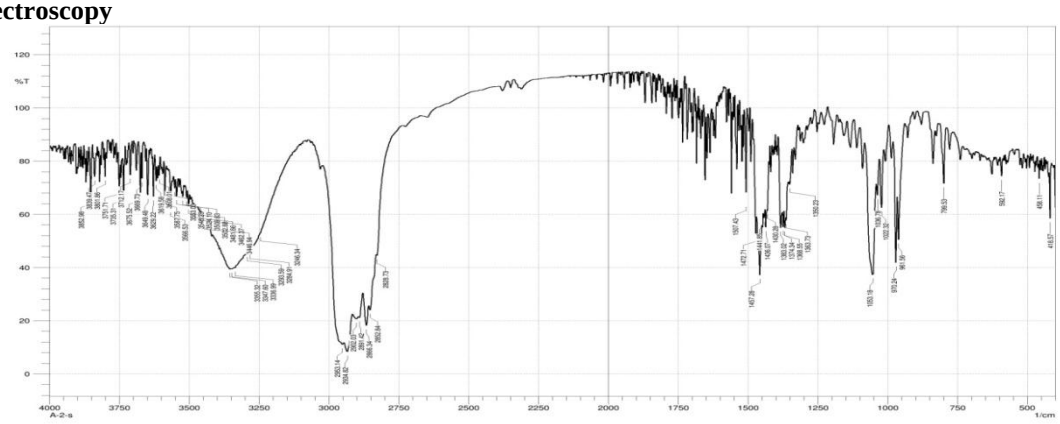

Fig. 4: FT-IR spectra of standard stigmasterol.

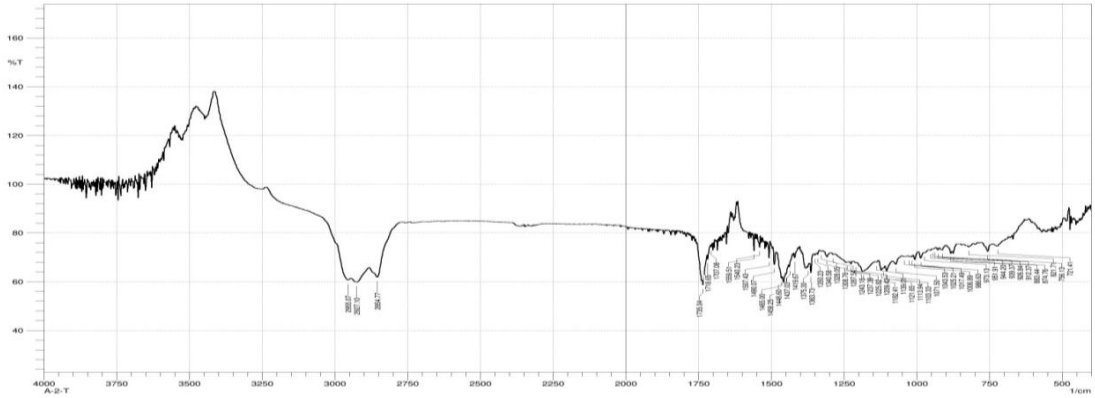

Fig. 5: FT-IR spectra of isolated stigmasterol.

Table 4: FT-IR data of standard and isolated stigmasterol.

S. No.	Standard Stigmasterol Frequency cm^{-1}	Isolated compound Frequency cm^{-1}	Assigned grouping
1.	799.53	821.71	=C-H
2.	970.24	973.13	=C-H
3.	1053.81	1043.53	C-O
4.	1457.28	1458.25	C-H
5.	2934.82	2927.10	O-H Stretch

UV Spectroscopy

λmax determination of isolated stigmasterol

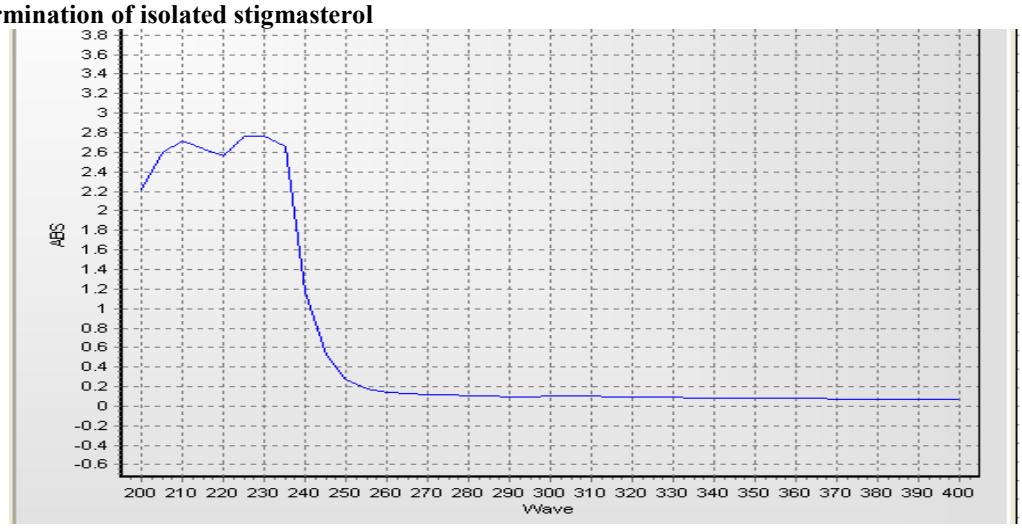

Fig. 6: UV Survey Scan of stigmatsterol.

Calibration curve of isolated stigmasterol

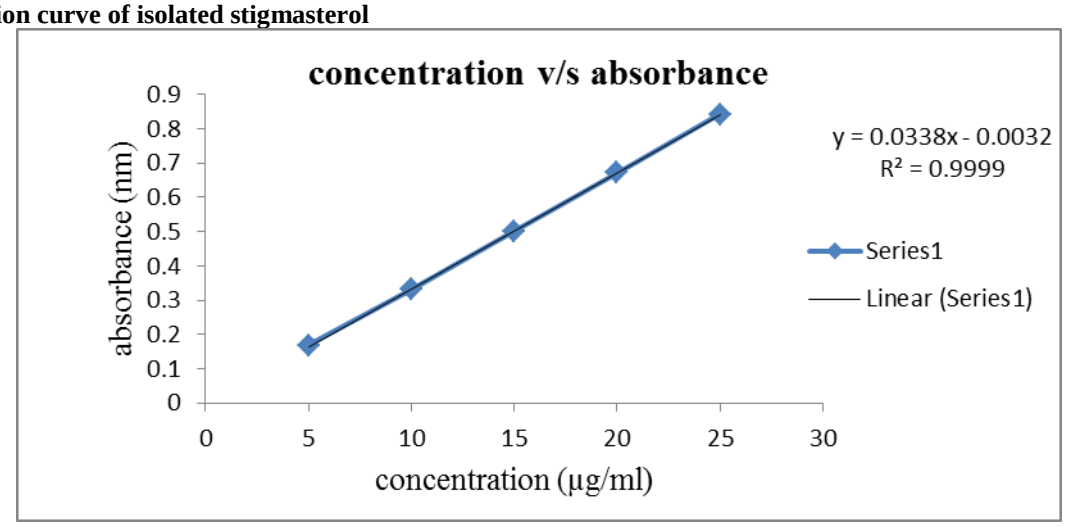

Fig 7: Calibration curve of stigmasterol.

RP-HPLC method development

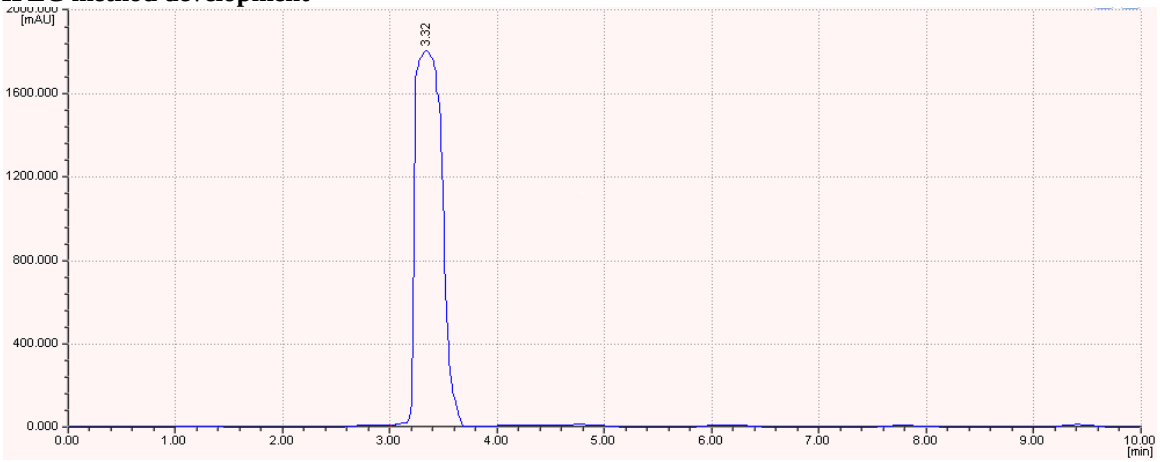

Fig. 8: HPLC chromatogram of standard stigmasterol.

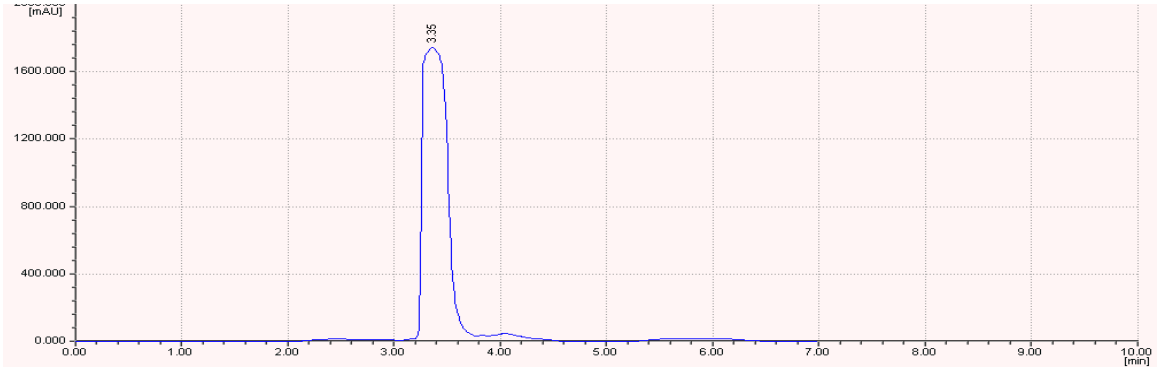

Fig. 9: HPLC chromatogram of isolated stigmasterol.

Method Validation

Linearity

Table 5: Linearity range determination of the developed HPLC method of stigmasterol.

S.no.	Concentration (µg/ml)	Area under the curve (AUC)
1.	2	51127
2.	4	100785
3.	6	151768
4.	8	201825
5.	10	270500
Slope		**26989**
R^2		**0.995**
Intercept		**6734**

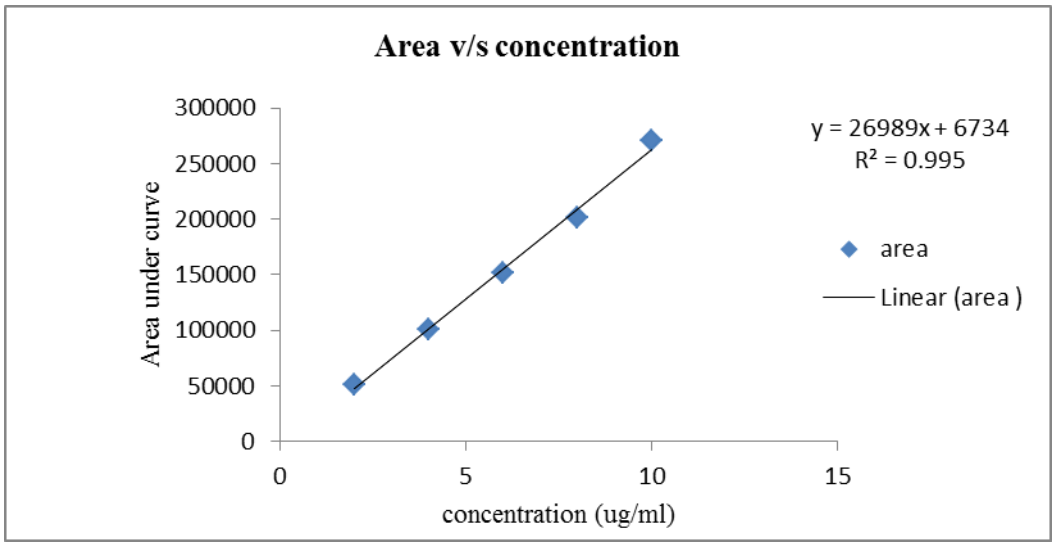

Fig 10: linearity range curve of stigmasterol.

Accuracy

Table 6: Accuracy data for area under the curve (AUC) of the Developed HPLC method of stigmasterol.

S. no.	Concentration	AUC
1.	2	51145
2.	2	51540
3.	2	52195
4.	2	51280
5.	2	51346
Mean ±S.D.		**51501.2 ± 413.146**
%RSD		**0.802**

Precision

Intra-day precision

Table 7: Intra-day precision data for peak areas of the developed HPLC method of stigmasterol.

S. No.	Concentration	AUC
1.	6	150948
2.	6	151250
3.	6	151506
4.	6	152896
5.	6	152994
Mean ± S.D.		**151918.8 ± 958.008**
% RSD		**0.630**

Inter-day precision

Table 8: inter-day precision data for peak areas of the developed HPLC method of stigmasterol.

S. no.	Concentration	AUC
1.	6	150724
2.	6	151115
3.	6	151606
4.	6	152355
5.	6	152702
Mean ±S.D.		**151700.48 ± 827.1434**
% RSD		**0.545**

Robustness

Table 9: Robustness data at different flow rate and λ_{max} for peak areas of the developed HPLC method of stigmasterol.

Sr. no.	Condition	Concentration (µg/ml)	Area	Mean ± S.D.	%RSD
1	At λmax 210	6	150946 152247 152792	151995 ± 948.449	0.624
2	At λmax 212	6	150635 152206 152492	151777.66 ± 999.857	0.658
3	Flow rate at 1.2ml/min	6	151998 153905 152886	152951.57 ± 953.57	0.623
4	Flow rate at 1 ml/min	6	151950 153880 152958	152915.07 ± 965.07	0.631

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Table 10: LOD and LOQ data of developed HPLC method of stigmasterol.

Sr. no.	Parameters	Conc. In µg/ml
1	LOD	0.914 (µg/ml)
2.	LOQ	2.77 (µg/ml)

CONCLUSION

A simple, specific, precise, rapid, and reproducible HPLC method has been developed to stigmasterol using relevant marker compounds in extracts of *Ocimum sanctum* leaves. The isolated compound (stigmasterol) had potential of anti-diabetic as comparable to standard metformin.

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