SUPPLEMENTARY MATERIAL

Development and validation of RP-HPLC method for the determination of stigmasterol in the botanical extract of Ficus deltoidea

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Experimental

Chemical and standards

Stigmasterol standard was purchased from Otto chemie Pvt ltd Mumbai. Methanol, chloroform and acetonitrile were of HPLC grade and obtained from Merck. All other chemicals used in the study were of analytical grade and obtained from Merck.

Plant material and extraction procedure

Plant leaves were collected from the Botanical Garden, Khalsa College, Amritsar in the month of April. The leaves were identified taxonomically by Professor (Dr.) Parveen Kumar Ahuja, Senior Taxonomist, Department of Life Sciences, Khalsa University Amritsar. The voucher specimen (LSKU/101-18022017) of authenticated plant material was deposited at Khalsa college of Pharmacy, Amritsar for future reference. The procured leaves were rinsed with water, air dried and pulverized to get coarse powder.

The completely dried coarse powder of FDL was subjected to soxhlet extraction with petroleum ether for 48 hour. The solvent was recovered from the crude extract under reduced pressure to obtain dark green oily material and labeled as crude petroleum ether extract. This extract was then allowed to be saponified with alcoholic potassium hydroxide to remove most fatty material and the remaining unsaponified fraction was collected with chloroform and allowed to dry at room temperature for 3- 4 days.

Preparation of stock and working standard solutions

A stock solution (100 µg/ml) of stigmasterol was prepared by mixing 1 mg of standard stigmasterol in 10 ml of methanol and allowed to sonicate for 10 min. Working standard solutions of stigmasterol were prepared by diluting 0.2, 0.4, 0.6, 0.8 and 1.0 ml of stock solution in 10 ml of methanol to furnish five different concentration of working standard i.e. 2, 4, 6, 8 and 10 µg/ml.

Preparation of sample stock solution

The stock solution of plant sample was prepared by dissolving 50 mg of the dried chloroform fraction in 50 ml of methanol. The sample was sonicated for 10 min than filtered using whatman filter paper. From this solution, the aliquot of 1 ml was transferred into the flask of 10 ml and filled to the mark with methanol. Further 2 ml solution was transferred into flask and volume made upto 10 ml using methanol (20 µg/ml).

HPLC method of analysis

HPLC analysis was carried out on Agilent 1260 infinity HPLC system. The system was equipped with photodiode array detector and was set to monitor at 200 - 400 nm. The separation was achieved with Agilent Zorbax XDB-C18, 250 mm×4.6mm×0.5 µm column thermostated at 25^{o}C. An isocratic method with acetonitrile (75%) and 0.1% acetic acid in water (25%) was used for the separation of peaks for the duration of 20 min. Sample volume was 20 µl and flow rate of mobile phase was adjusted to 1.0 ml/ minute. Peaks were integrated at the wavelength of 210 nm.

Method validation

Validation is essential to determine the variability in the developed HPLC method and its control. Validation of analytical method for the determination of stigmasterol in chloroform fraction of *Ficus deltoidea* leaves was done by accessing different parameters such as linearity, accuracy, precision, sensitivity and robustness as per ICH regulatory guidelines.

Linearity and range

The linearity of the method was evaluated by constructing calibration curve with five different concentrations of stigmasterol working standard solutions (2, 4, 6, 8 and10 µg/ml). The analyte was analysed in triplicate, and the calibration curve was constructed by plotting the area under the curve of the sample main peak versus concentration of analyte. The linearity was evaluated by determining the correlation coefficient, Y- intercept and slope of regression line.

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) values were calculated from the calibration curves of stigmasterol as k (σ/s) where k=3 for LOD and 10 for LOQ. σ is the standard deviation of the response of the minimum detectable drug concentration and s is the slope of calibration curve.

Accuracy

The accuracy of method was evaluated by recovery assay using addition method. The assay was carried out by adding known concentration of standard in pre-quantified stigmasterol solution (2 µg/ml and 4 µg/ml) to reach the 80, 100, 120% levels. The assay was performed in triplicate to determine the average percentage recovery.

Precision

Precision describes the variation in results observed after repeated analysis of the sample under identical experimental conditions. The method was validated by evaluating the intra- and inter-

day precision. The intra-day and inter-day precision were measured from triplicate injection of five sample solutions of targeted concentration of 6 µg/ml under identical experimental condition in a single day or performed on three different days respectively. Precision assay was expressed as relative standard deviation (RSD) which was calculated from the observed data. To determine the intraday precision or repeatability of method, five injections of targeted concentration were done on same day. Similarly, the experiment was repeated on three consecutive days by different individuals to determine inter-day precision.

Robustness

Robustness of the proposed analytical method was determined by varying the chromatographic conditions. The λ max (wavelength) of the sample was changed from 205 to 215 nm and flow rate of the mobile phase was changed from 0.8 ml/min to 1.2ml/min. Triplicate standard solution of targeted concentration (6 µg/ml) was injected onto column and response was observed.

Statistical analysis

The data of each parameter was recorded as mean of three experiments and its statistical analysis was performed in MS-Excel 2007.

Table S1: Results of linearity study for stigmasterol

Sr. no.	**Conc. µg/ml**	**Area**
1	2	12682.2
2	4	28560.4
3	6	42723.0
4	8	59896.8
5	10	72645.2
Slope	7563.12	
r^2	0.998	
Intercept	-2077.2	

Table S2- Analytical recovery of stigmasterol standard solution added to known concentration of sample

Amount present (µg/ml)	% level of recovery	Amount added (µg/ml)	Amount found (µg/ml); n=3	%Recovery	Mean % RSD
2	80	1.6	3.601	100.01	0.16
2	100	2.0	3.986	99.66	0.11
2	120	2.4	4.406	100.1	0.09
4	80	3.2	7.194	99.9	0.07
4	100	4.0	7.991	99.8	0.10
4	120	4.8	8.789	99.8	0.03

Table S3: Analysis of intra-day and inter-day precision assay

Precision	Conc. (µg/ml)	Area	Mean ± SD	% RSD
Intra-day precision	6	43309.1	43304.64 ± 115.32	0.266
	6	43157.8		
	6	43235.2		
	6	43225.4		
	6	43395.7		
Inter-day precision	6	45895.6	45813±115.98	0.340
	6	45598.2		
	6	45702.7		
	6	45908.2		
	6	45965.1		

Table S4-Robustness study of HPLC method

Condition	Conc. µg/ml	Area	Mean ± SD	%RSD
λmax 205 nm	6	45302.8 45109.2 45232.8	45214.93 ±98.03	0.216
λmax 215 nm	6	45797.5 45710.8 45586.4	45698.23±106.11	0.232
Flow rate 0.8ml/min	6	45852.9 45609.6 45759.8	45740.77±122.76	0.268
Flow rate 1.2 ml/min	6	40571.3 40508.2 40815.8	40631.77±162.47	0.399

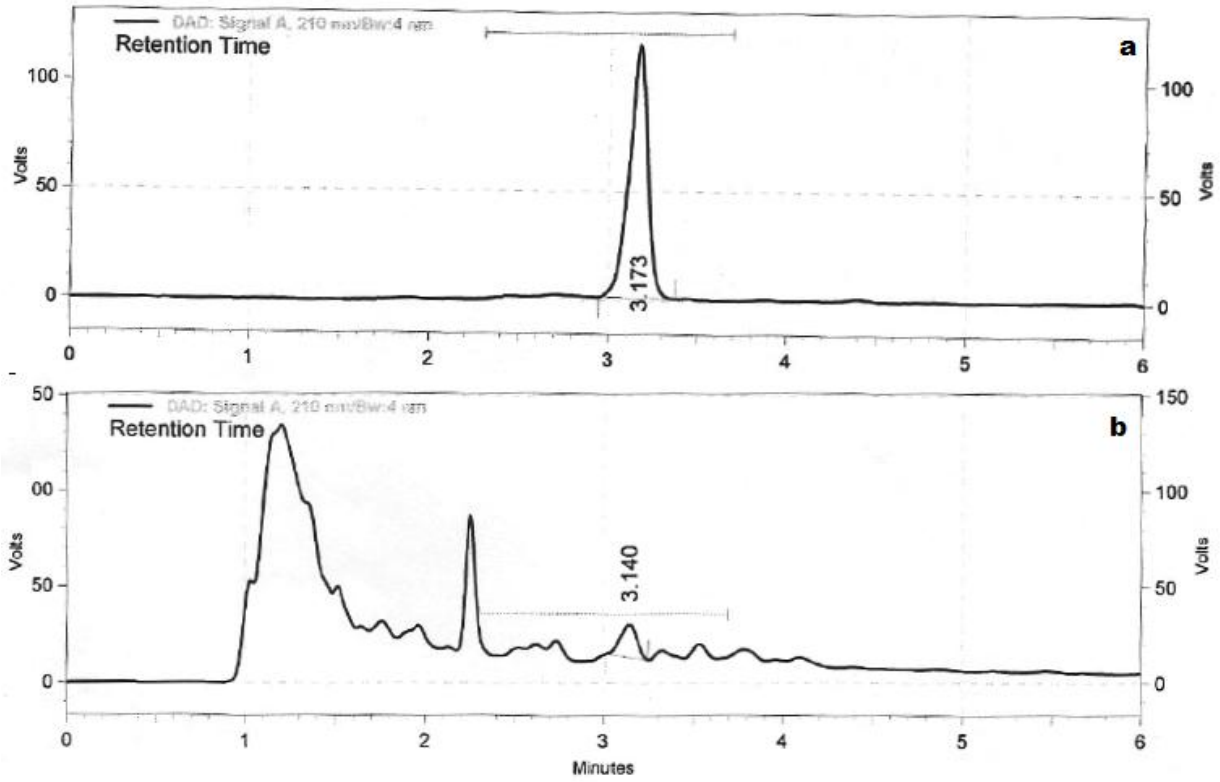

Figure S1: HPLC chromatograms of standard stigmasterol (a) and chloroform fraction of *Ficus deltoidea* leaves (b).

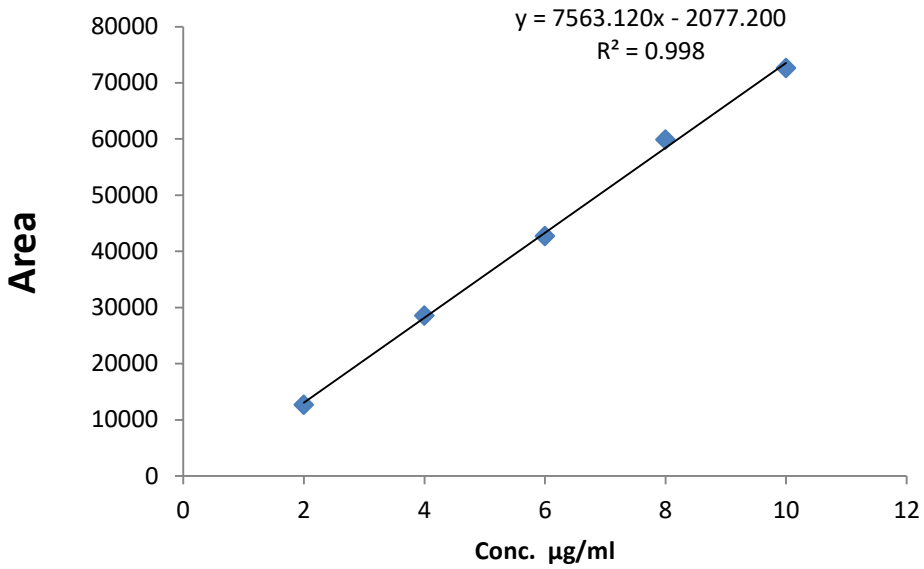

Figure S2: Calibration curve of stigmasterol

Contents