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Development and validation of HPLC methods for the standardization of Stigmasterol & Lupeol from the extract of *Butea monosperma* (Lam) and it's formulation

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Abstract
The determination of natural compounds Lupeol and Stigmastrol in plant extracts using HPLC Shimadzu model Lc20AD AHT to manual sampler (UV-SPD-20A Detector) is reported. The methods were applied to the analysis of Lupeol and Stigmastrol in petroleum ether extract originating of *Butea monosperma* (Lam) bark. The method was validated using ICH guidelines in terms of precision, repeatability, recovery and accuracy. Regarding HPLC method validation, the optimize mobile phase system used was Methanol: Water (98:2%v/v) with the 1ml/min flow rate and the detection wavelength was 220nm.

Keywords: *Butea monosperma* (Lam.), Lupeol, Stigmastrol, HPLC

Introduction
Butea monosperma (Lam.) (Syn-Butea fondosa) belonging to the family Fabaceae. This tree generally known as "PALAS", "DHAK", or "FIRE OF FOREST". Orange colours of flowers are main identical characteristic of this tree. Very widely even distributed in India, Burma, Myanmar, Nepal, Ceylon and moderate in size. The bark consist of Kino-tannic acid, palasitrin, and major glycosides as butrin, alanind, allophanic acid, butolic acid, cyanidin, histidine, lupenone, lupeol, (-)-medicarpin, miroestrol, palasimide and shellolic acid. This plant is traditionally reported to possess alterative, anthelmintic, antibacterial, astringent, aphrodisiac and diuretic [1, 2, 3].
Now days traditional systems of medicine have been explore in current global drug market. Quality control and Standardization both are most important aspect for the herbal drug formulation. Generally Herbal Formulations are based on polyherbal formulation. Plant based drugs are extracted, isolated and purified for their therapeutic utility based on their selective pharmacological activity. Standard markers are use quantitative and qualitative analysis for herbal drug formulation. Lack of proper standard parameters and methods for the standardization of herbal formulation and preparation has led to several instances of substandard herbs and adulterated herbs coming into existence [4, 5, 6].
Up till now, has not been reported for simultaneous estimation of Lupeol and Stigmastrol from *Butea monosperma* (Lam.). In this paper development and validation of a HPLC method for the quantitative analysis Lupeol and Stigmastrol is reported. The proposed method has been validated as per ICH guidelines [7, 8, 9].

Method and Materials
Reference standards and reagents
The reference standards Lupeol (purity >95%) and Stigmasterol (purity > 95%) were purchased from Natural Remedies Pvt. Ltd. Bangalore and HiMidia Laboratories Pvt. Ltd. Mumbai. Distilled water was prepared with a Milli-Q academic water purification system (Millipore, Bedford, MA, USA). Methanol (HPLC grade) was purchased from Avantor performance materials India Limited, Mumbai, India. Before use, all the solvents were filtered through membrane filters Nylon 66 of 0.2 mm pore size (Millipore).

Plant materials collection and extraction
Butea monosperma (Lam.) was collected from herbal garden, Faculty of Pharmacy, The M.S. University, Vadodara .This plant sample authenticated by Dr.P.S.Nagar, Botany department of The M.S. University. Dried plant material (300gm.) was powdered and extracted/defatting with petroleum ether in Soxalet apparatus (60- 70C) for 48 hour and the solvent evaporated to dryness in rotary evaporator, yielding 0.52% (W/W) crud extract.

Compound isolated by the column chromatography using 2% (v/v) methanol: chloroform.

Instrumentation and analytical conditions

Uplc Model- Shimadzu, Sampler-Manual, Detactor-UV PDA-20AV, Software- Springcom (LC-Solution), Pump-Lc20AV AHT

Detection wavelength: 220nm Flow rate: 1ml/min Temperature: Ambient

Mobile phase: Isocratic- Methanol: Water (98:2% v/v)

Column used: Phenomenex, Luna C18 column (150×4.6, 5µ)

Standard solution preparation

Stock solutions of reference standards lupeol (1mg mL-1) and stigmasterol (1 mg mL-1) were prepared in methanol (HPLC grad). Appropriate amount of each standard stock was mixed separately to prepare working standard solutions containing six different concentrations of lupeol and stigmasterol (10, 50, 100, 200, 400 and 800 µg mL-1) for establish the calibration curves. Standard solutions contain lupeol and stigmasterol (100, 200 and 400 µg mL-1) were subjected for method validation. All solutions were stored at 4 °C prior to analysis.

Sample preparation

Accurately weighted 10 mg solvent free dried extract was dissolved in 5 mL methanol to prepare concentration of 1 mg mL-1. The aliquot was then filtered through 0.45 µm membrane filter prior to injection.

Calibration curve

The calibration curves were established by analysing (n = 6) the six different concentrations of each reference standard at concentrations ranged from 5 - 200 µg mL-1 for lupeol and and from 10 - 800 µg mL-1 stigmasterol, respectively. Calibration curves were constructed by plotting the peak areas versus the concentrations of each standard by means of linear regression.

Method validation

The developed UFLC method for simultaneous quantitative analysis of Lupeol and Stigmasterol was validated in term of linearity, specificity, system suitability, limits of detection (LOD) and quantification (LOQ), accuracy, precision, robustness and ruggedness. Validation of the method was performed as recommended by the International Conference on Harmonization (ICH) guidelinesQ2R1.

Statistical analysis

The results were statistically analysed using Graph Pad Prism version 5.0. The results were calculated as the mean ± SD/SEM.

Results

Optimization of chromatographic conditions

In this method development such chromatographic conditions are matters like mobile phase proportion; flow rate, column grad, and detection wavelength were optimized to provide sufficient selectivity and sensitivity. Batter separation and good peak resolution mobile phase composition Methonal: Water (99:2%v/v) was selected for separation. Further, column 250×4.6mm SS EXSIL ODS was used with optimised mobile phase. Pump flow rate increase with 0.1- 1.4ml $min^{-1.}$ Ideal pump flow rate of compound mixture was 1ml $min^{-1.}$ When flow rate increased simultaneously retention time of compound mixture decreased. Lupeol and Stigmasterol detected wave length at 220 nm. Best peak resolution was observed at 25 ºC. Using the optimized conditions, all marker compounds were successfully resolved and eluted within 15 min. all process happen under isocratic mode.

Method Validation

System Suitability Linearity

Linearity achieve by the good concentration range. Stigmasterol and Lupeol standard mixture linearity concentration range was 0.2µg mL-1 to 0.5µg mL-1. The regression equations and correlation coefficient for the reference were y = 3E+06x - 150447

R^2 = 0.9964 for Lupeol and y = 2E+06x + 57707

R^2 = 0.998 for Stigmasterol respectively. The experiment was performed three times and the mean was used for the calculations. The data was analyzed by linear regression least squares fitting.

Limit of Detection and Limits of Quantitation

The LOD and LOQ values were calculated based on the ICH guidelines [R1 Q2], by determining the SD of the response and the slope of the linear equation. LOD and LOQ under proposed chromatographic conditions were calculated using the formula: LOD = 3.3σ/S and LOQ = 10σ/S. Where, σ is the standard deviation of the response from a number of blank run and S is the slope of the calibration plot. The LOD values of Lupeol and stigmasterol were 0.007 and 0.0385 µg mL-1, respectively and their respective LOQ values were found to be 0.021 and 0.11 µg mL-1.

Assay

Precision and Accuracy

The accuracy of the method was evaluated by recovery study. The recovery study was performed by addition of known amounts of each standard to the pre-analysed sample (n = 3) followed by the reanalysis of the contents using the developed method. The recovery data revealed that the mean recovery values of three different concentrations of Lupeol and Stigmasterol were and respectively

Table 3: Recovery study of Lupeol and Stigmasterol from synthetic mixture (n = 3)

Reference standards	Amount added (µg)	Total content (µg)	Amount found (µg)	Recovery (%)	Mean recovery (%)
Lupeol	80%	0.32	0.8	97.64%	96.80%
	100%	0.4	0.88	100.50%	
	120%	0.48	0.96	92.25%	
Stigmasterol	80%	0.32	0.54	97.19%	97.20%
	100%	0.4	0.62	98.17%	
	120%	0.48	0.7	96.25%	

System suitability

System suitability was analyzed in terms of peak area, RT, tailing factor (must be < 2), theoretical plate count (should be > 20000) etc. For system suitability, six replicates (n = 6) of standard solution containing betulin (100 µg mL-1), lupeol (400 µg mL-1) and stigmasterol (400 µg mL-1) were analysed to establish %RSD of RT, peak area, tailing factor and theoretical plate count.

Table 4: System suitability data of the proposed method (n = 6)

Reference standards	RT	%RSD	intraday	%RSD	interday	%RSD	Tailing factor	%RSD
Lupeol	8.7038	0.1712	512930.2	1.237337	649949.6	2.05385	1.035556	0.343059
Stigmasterol	12.40156	0.2591	395669.7	0.826579	439608.2	0.026531	1.024	1.5677

Robustness

The robustness of the proposed method was determined by analyzing (n = 6) the standard solutions of Lupeol (µg mL-1), and Stigmasterol (µg mL-1) under small changes in the optimum conditions set for this method such as flow rate, detection wavelength, wave length and column temperature. Under the modification of such critical parameters, no significant changes were observed in the RT, peak area response and recovery of the standard compounds with %RSD values of less than 2%.

Validation Parameters

Parameters	HPLC	
	Lupeol	**Stigmasterol**
Linearity (ng/spot)	01-Oct	01-Oct
Analytical wavelength (nm)	220	220
Regression equation	y = 3E+06x - 150447	y = 2E+06x + 57707
Intercept	24309.62	23354
Slope	2333333	2000000
(r^2) Regression equation	0.999	0.998
LOD	0.007	0.0385
LOQ	0.021	0.11
Precision(%RSD)		
Intraday	1.237337	0.826579
Interday	2.05385	0.026531
Accuracy (% recovery)	96-102%	96-102%
ASSAY	4.80µg/ml	2.52µg/ml
Stability on plate (min)	5	

Conclusion

Standardization of the selected medicinal plant *Butea monosperma* and its poly herbal formulation were carried out by UFLC methods. In the current work the method was found to be simple, accurate and precise. Hence these are recommended as they procedures are well suited for the estimation of stigmasterol and lupeol in its marketed formulations.

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