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Quantification Of Lupeol Isolated From Strobilanthus callosus Nees using Validated High Performance Thin Layer Chromatography Method.

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Abstract: *Strobilanthes callosus* Nees *(Synonym: Carvia callosa (Nees) Bremek)* is a shrub found mainly in the lower hills of the western ghats all along the west coast of India. A sensitive high performance thin layer chromatographic method has been established for quantification of Lupeol which was isolated from dried stem powder of *Strobilanthus callosus* Nees. Chromatographic separation was performed on TLC alumina plates precoated with silica gel $60F_{254}$ as the stationary phase with toluene: ethyl acetate: glacial acetic acid (6.0:1.5:0.1v/v/v) as the mobile phase. The plates were scanned densitometrically in the reflectance absorbance mode at 580 nm. The method was validated for linearity, precision, limit of detection (LOD), limit of quantitation (LOQ), and accuracy. Linearity was found to be in the range of 40.00µg/mL to 100.0µg/mL with value of correlation coefficient (r) = 0.9986. Instrumental precision, repeatability and intermediate precision were determined to evaluate the precision of the method. The accuracy of the method was established by evaluating % recovery of Lupeol at three different levels and it was found to be 98.59. The proposed parameters in the paper may help to establish the authenticity of the drug and drawing the pharmacopoeial standards for this specimen.
Keywords: *Strobilanthes callosus* Nees**;** Lupeol; HPTLC; Method Validation; Acanthaceae.

INTRODUCTION

The genus Strobilanthus belonging to the family Acanthaceae has around 250 species, of which at least 46 are found in India. *Strobilanthes callosus* Nees *(Synonym: Carvia callosa (Nees) Bremek)* is a shrub found mainly in the lower hills of the western ghats all along the west coast of India[1] (Fig. 1). Its standardized Hindi language name is Maruadona by which it is called in the state of Madhya Pradesh. In the state of Maharashtra in the Marathi language and other local dialect and in the neighboring state of Karnataka the shrub is locally known as Karvi, sometimes written in English as Karvy[2]. This shrub belongs to the genus *Strobilanthes* which was first scientifically described by Nees in the 19th century[3].

Figure No. 1: Plant of Strobilanthus callosus

The stem bark of *Strobilanthes callosus* is used as an emollient in formulations for painful and ineffectual attempts to urinate or defecate[4]. It is used externally for mumps and flowers, used as a vulnerary[5]. Likewise, pounded leaves are rubbed on to the body during the cold period of an intermittent fever and used as a poultice to treat ague in children to alleviate coughing, also used as an astringent and diuretic[6]. The root is used in treatment of inflammation and arthritis[7]. The roots contain Lupeol and have been examined by influorescence for volatile oil-borucol, sitosterol, hexacosane, campesterol and others[8]. The Chinese Herbal formulation 'Shengma' was formulated through the combination of Serratulla and Strobilanthes which had a considerable effective action as an anti inflammatory drug[9]. But there are no literature reports of isolation of Lupeol from the stem bark of *Strobilanthes callosus* Nees hence, this study was carried out which encompasses the isolation, method development and method validation for quantification of Lupeol isolated from stem bark of *Strobilanthus callosus* Nees.

EXPERIMENTAL METHODS

MATERIALS

STANDARD AND REAGENTS

Lupeol (purity 98%), was gift sample from Natural Remedies, Bangalore, India. Ethyl acetate, toluene, glacial acetic acid, methanol and sulphuric acid used in the present research work were of HPLC grade and were procured from E. Merck Mumbai, India.

PLANT MATERIAL

Strobilanthes callosus Nees was procured from Trimbakeshwar, Nasik, India and authentificated in Botanical Survey of India (BSI) and voucher specimen (RSC-1) was kept at departmental herbarium of BSI, Pune. Drug material (stem) was powdered and then sieved through BSS mesh size 85 and stored at 25ºC, in an airtight container.

EXTRACTION, ISOLATION OF LUPEOL FROM STROBILANTHUS CALLOSUS

Nearly about 5 kg of the dried drug material was undergone successive extraction with solvent of increasing polarity like pet ether, chloroform and methanol (48hours each solvent). The yield obtained for pet ether was 98.2gms for 5 kg material. The percent yield obtained was 1.97% w/w. Out of this; 50 gm of dried pet ether extract was then saponified with alcoholic KOH to remove the fatty material, yielded nearly about 22 gm of unsaponified material. Nearly about 12 gms of unsaponified matter were subjected to column chromatography on silica gel (60-120 mesh) as a stationary phase. Gradient elution was performed using Toluene: Methanol (10:0; 9:1 up to 0:10) as the mobile phase. 120 fractions were collected in the test tube. On evaporation of mobile phase' pure white crystals were obtained in the test tubes of Toluene: Methanol (9:1) fractions. Single spot was resolved at Rf 0.70 using Toluene: Methanol (9:1) as mobile phase. The spot resoled was dark violet in colour. A total of 0.82 gms (820 mg) Lupeol were isolated. This isolated component was characterized as Lupeol and was used as a test compound for analysis.

PREPARATION OF STANDARD SOLUTIONS OF LUPEOL

Stock solution of Lupeol (1000.0 µg/ mL), was prepared in 10 mL standard volumetric flask, by dissolving 10.0 mg of accurately weighed Lupeol, in about 5.0 mL of methanol, followed by vortex and finally making up the volume of solution to 10.0 mL, with methanol. 5.0 mL of the above stock solution was diluted to 10.0 mL, with methanol to give standard solution of Lupeol, with concentration of 500.0 µg/mL. The aliquots (0.2 mL to 2.8 mL) of 500.0µg/mL solution of Lupeol, were transferred to 10.0 mL volumetric flasks and the volume of each flask was made upto 10.0 mL, with methanol, to obtain the working standard solutions of Lupeol, in the concentration range of 10.0 µg/mL to 140.00 µg/mL.

INSTRUMENTATION

CHROMATOGRAPHIC CONDITIONS

The samples were spotted in the form of bands of width 6mm and 12.2 mm apart with a Camag microlitre syringe on precoated silica gel aluminium Plate $60F_{254}$ having 20 cm × 10 cm dimensions (E. Merck, Darmstadt, Germany) using a Camag Linomat IV sample applicator (Muttenz, Switzerland). The mobile phase consisted of toluene: ethyl acetate: glacial acetic acid, in the volume ratio of 6:1.5:0.1. Linear ascending development was carried out in 20 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature. The length of chromatogram run was 80mm. Subsequent to the development, TLC plate was sprayed with methanol sulphuric acid reagent followed by drying in oven at 110ºC. Densitometric scanning was performed on Camag TLC scanner III in the reflectance absorbance mode at 580 nm and was operated by CATS software (V 3. Camag).

METHOD VALIDATION

LINEARITY

The calibration curve was set from the lower limit of quantification (LLOQ) to the higher limit of quantification (HLOQ). The linearity of the method was tested by applying standard Lupeol solution from 10.00 µg/mL to 140.00 µg/mL to silica gel alumina plates using above chromatographic condition. The densitograms were recorded and the peak areas of Lupeol for each applied concentrations were noted. The response factors were calculated for each concentration of Lupeol by dividing peak areas by corresponding concentration of Lupeol. The results indicated in Table 1.0, show that within the concentration range indicated, there was a good correlation between mean peak area and concentration of Lupeol.

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

The limit of detection is the lowest limit at which the analyte of interest is detected and determined at a signal to noise ratio of 3:1. The limit of quantitation is the lowest limit at which the analyte of interest is quantified with suitable precision and accuracy and is determined at a signal to noise ratio of 10:1. The LOD and LOQ values obtained are listed in Table 1.

PRECISION

The method was validated in terms of instrumental precision , intra assay precision and intermediate precision. The instrumental precision was studied by analyzing the standard solution of Lupeol of concentration 60.000 g/mL, in ten replicates, in the chromatographic system under the specified conditions. The intra assay precision is the precision which is obtained by using different concentration samples under the same applied chromatographic conditions on the same day and it was studied by analyzing three different concentration of sample solution of *Strobilanthus callosus* in triplicates. The Intermediate precision of the method was evaluated by analyzing the sample solution in triplicates on three different days, in the chromatographic system, under the same specified conditions. The results are expressed as % R.S.D. of peak area of Lupeol. The results are listed in Table 1. The results indicated that the method is precise and reproducible and gives good precision and accuracy resulting in quantification of Lupeol.

SYSTEM SUITABILITY

System suitability was carried out to verify that the resolution and reproducibility of the system were acceptable for the analysis. System suitability test was carried out by applying 10µL of standard solution of Lupeol of concentration 60.00 µg/mL on TLC plate in five replicates and analyzing under specified chromatographic conditions. The parameters used to determine system suitability were repeatability of peak areas and retention factor of Lupeol for replicate analysis. The values of mean peak area of Lupeol was found to be 1035 and retention factor (Rf value) of Lupeol was found to be 0.48 respectively with % R.S.D. value less than 2.

Table No. 1: Method validation parameters for the estimation of Lupeol by the proposed HPTLC method

Parameter	Results
Linear range µg/mL (n = 5)	40.00 to 100.00 µg/mL
Correlation coefficient (r)	0.9986
LOD µg/mL	10.00
LOQ µg/mL	40.00
Instrumental precision% R.S.D. (n=10)	0.38
Intraassay precision % R.S.D. (n=3)	0.44
Intermediate precision % R.S.D. (n=3)	0.54

VALIDATED METHOD APPLICATION FOR THE QUANTIFICATION OF LUPEOL FROM STEM OF *STROBILANTHUS CALLOSUS*

For the purpose of quantification of Lupeol from stem powder of *Strobilanthus callosus* accurately weighed 1.002g dried powder was taken in a dry, clean stoppered test tube (capacity 20 mL). To the above stoppered test tube, 10mL of methanol was added and the test tube was shaken at 20 rpm, on a rotary shaker at room temperature (28ºC ± 2ºC) for 12 hrs. The contents of tube where then filtered through Whatman filter paper No. 41. The filtrate obtained was used as sample solution for carrying out the experiment of quantification of Lupeol . The quantification of Lupeol was done using above validated HPTLC method. 10µL of the sample solution was applied as bands in seven replicates on the TLC plate, with a Camag Linomat IV sample applicator and analyzed using optimized chromatographic conditions. The identity of peak of Lupeol in the sample solution was confirmed by comparing the retention factor (Rf) value of the sample with that of the standard solution of Lupeol having Rf value as 0.48. A TLC plate showing the Rf value of standard Lupeol and sample Lupeol is shown in Figure 2. A Typical Chromatogram of Standard Lupeol under above mentioned chromatographic condition is represented in Figure 3. A Typical Chromatogram of plant extract sample showing the peak of Lupeol is represented in Figure 4. To ascertain the repeatability of the method, the assay experiment was repeated seven times. Mean amount of Lupeol in dried stem powder of *Strobilanthus callosus* was found to be 0.13mg/g.

Figure No. 2: High Performance Thin Layer Chromatography Separation of Standard Lupeol (A) and Sample Extract of Lupeol (B) from stem bark of *Strobilanthus callosus*

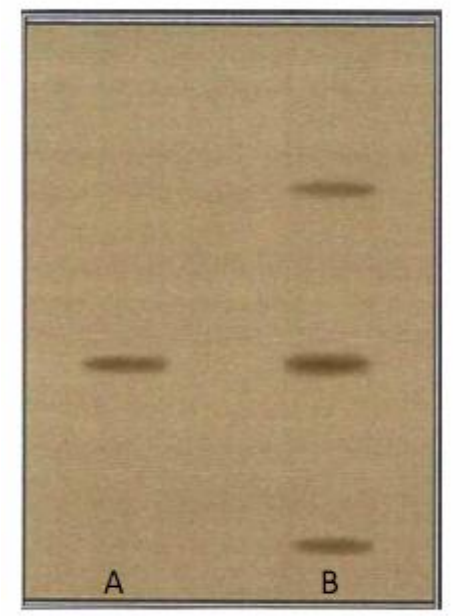
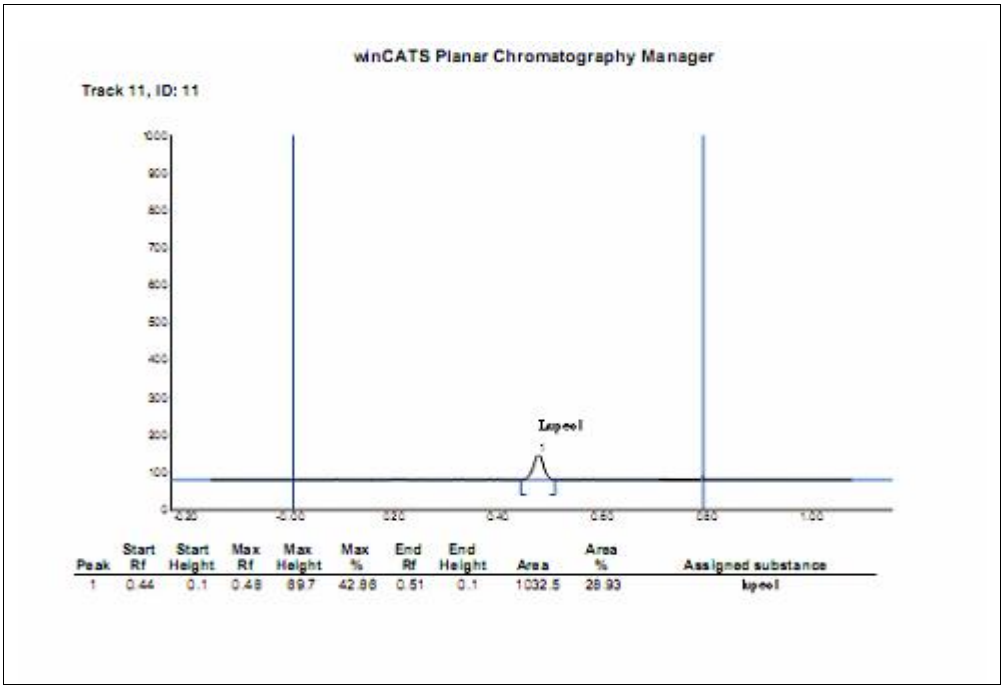
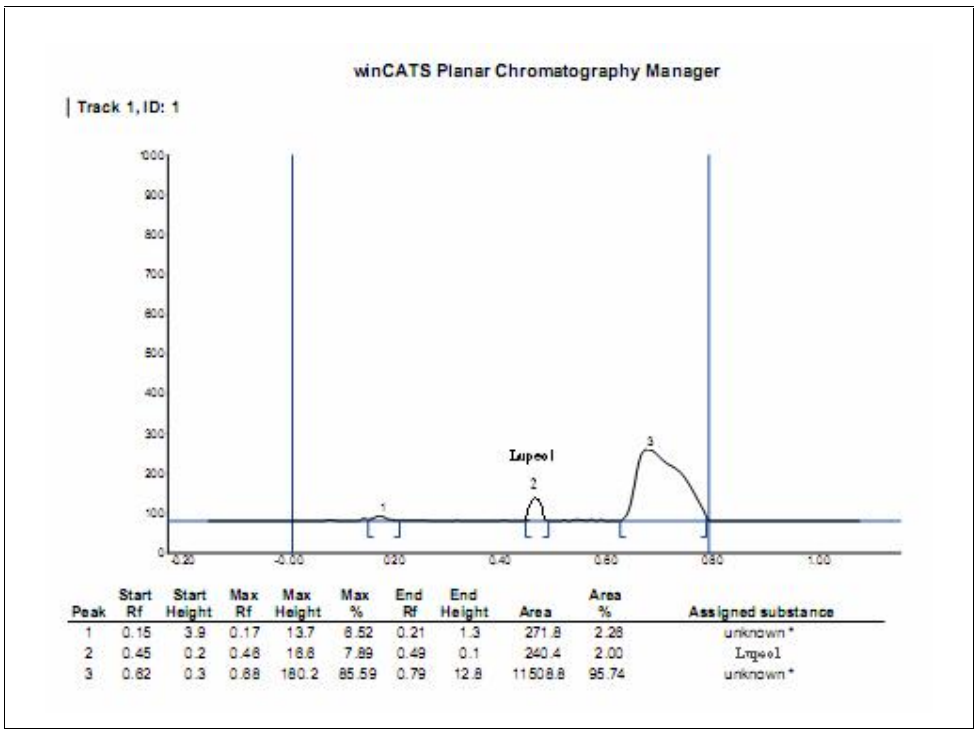


Figure No. 3: High Performance Thin Layer Chromatographic Determination of Standard Lupeol at the Rf 0.48



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.44	0.1	0.48	89.7	42.86	0.51	0.1	1032.5	28.93	lupeol

Figure No. 4: High Performance Thin Layer Chromatography Determination of Lupeol isolated from stem bark of *Strobilanthus callosus* at the Rf 0.48



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.15	3.9	0.17	13.7	6.52	0.21	1.3	271.8	2.28	unknown *
2	0.45	0.2	0.48	16.8	7.89	0.49	0.1	240.4	2.00	Lupeol
3	0.62	0.3	0.68	180.2	85.59	0.79	12.8	11508.8	95.74	unknown *

ACCURACY

The accuracy of the method was established by performing recovery experiments, using the standard addition method in which the standards of Lupeol are being used at three different levels along with the sample solutions of interest i.e. (stem powder of *Strobilanthus callosus*). About 1.00g of powdered stem of *Strobilanthus callosus* was accurately weighed into each of the four stoppered test tubes. Known amounts (0.00 µg, 1 µg, 2 µg and 3 µg) of standard Lupeol were added in solution form to each of the stoppered test tubes respectively and volume made upto 10.00 mL methanol in each test tube. The stoppered test tubes were then shaken at 20 rpm on rotary shaker for overnight at room temperature (28°C ±2°C). The contents of the test tube were filtered separately and each solution was analyzed seven times, under optimized chromatographic conditions. Value of percentage recovery for Lupeol was then determined. The results of the recovery experiment are given in Table 2. The value of percentage recovery of Lupeol is 98.59, indicating good accuracy of the method.

SOLUTION STABILITY

The stability of standard Lupeol solution was determined by comparing the peak areas of Lupeol solution of concentration 70.00 µg/mL, at different time intervals, for a period of minimum 48 hrs, at room temperature. The results showed that the peak area of Lupeol almost remained unchanged (% R.S.D. was less than 2) over a period of 48 hrs, and no significant degradation was observed within the given period, indicating the stability of standard solution of Lupeol, for minimum 48 hrs.

RESULTS AND DISCUSSION

The identity of peak of Lupeol in sample solution was confirmed by comparing its Rf value with that of standard Lupeol (Rf 0.48). A good linear relationship was obtained for Lupeol , in the concentration range between the lower limit of quantification(LLOQ) and higher limit of quantification (HLOQ) of 40.00 µg/mL to 100.00 µg/ mL. The correlation coefficient obtained was of 0.9986. The average percent recovery of Lupeol at three levels was 98.59 (Table 2).The stability of standard solution of Lupeol was found to be minimum 48 hrs with % R.S.D less than 2. Quantification of Lupeol from stem of *Strobilanthus callosus* was conducted and the mean amount of Lupeol in dried stem powder of *Strobilanthus callosus* was found to be 0.13mg/g. The method was validated in terms of instrumental precision (% R.S.D: 0.38) , intra assay precision (% R.S.D: 0.44) and intermediate precision (% R.S.D : 0.54). The results gave % R.S.D less than 2 with the area and Rf value of the test component as 240.4 and 0.48 respectively, it indicated that the method is precise and reproducible and gives good precision and accuracy resulting in quantification of Lupeol. System suitability was carried out to verify that the resolution and reproducibility of the system was acceptable for the analysis of the specified method developed. In system suitability the values of mean peak area and retention factor (Rf value) of Lupeol was found to be 1035 and Rf 0.48 respectively with % R.S.D. value less than 2. Thus a very simple and precise method is being developed for the quantification of Lupeol from stem bark of *Strobilanthus callosus* Nees.

Table No. 2: Recovery of Lupeol from stem bark of *Strobilanthus callosus*

Level	Wt of sample *(g)	Wt of standard (µg)	Mean amount of Lupeol found (mg/g)**	Percent recovery [%]
0	1.002	0	0.127	98.59
1	1.005	1	0.132	
2	1.004	2	0.135	

Mean amount of Lupeol found in 1.0gm of sample = 0.131mg

* Sample: Dried stem powder of *Strobilanthus callosus*

* * Mean amount of Lupeol found

CONCLUSION

Very less work has been done on the plants belonging to the category of plietesials that bloom after long intervals. The term plietesial has been used in reference to perennial *monocarpic* plants (a sub tribe of *Acanthaceae* containing *Strobilanthes* and allied genera) that usually grow gregariously, flower simultaneously following a long interval, set seed, and die. The genus has around 250 species, of which at least 46 are found in India. A new HPTLC method has been developed and validated and used for the quantification of Lupeol which is isolated from the methanolic extract of dried stem powder of *Strobilanthus callosus* Nees. The HPTLC method for the determination of Lupeol was validated in terms of linearity, precision, accuracy, system suitability, recovery and sample stability. The developed HPTLC technique is precise, specific, accurate and shows remarkable stability indicating that can be used for the routine quality control analysis and quantitative determination of Lupeol from dried stem powder of *Strobilanthus callosus* Nees for further scientific work.

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