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# Simultaneous Estimation of Stigmasterol and Lupeol in *Adenanthera pavonina* using HPTLC Method

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ABSTRACT

*Adenanthera pavonina* , a well-known medicinal plant has been used traditionally to treat diarrhea and inflammation. Its medicinal properties result from its active phyto-constituents, including non-protein amino acids, sterols, dulcitol, and fixed oil. Stigmasterol (STIGMA) and lupeol (LPL) are significant phytosterols in the plant's bark as they possess anti-inflammatory activities that may be used in multiple therapies. To design an accurate HPTLC method for the simultaneous estimation of lupeol and stigmasterol from the bark of *Adenanthera pavonina*. The STIGMA and LPL were isolated using silica gel (60–120 #) column chromatography and estimated using HPTLC. The TLC aluminium plates were pre-coated with silica gel $60F_{254,}$ the mobile phase comprised toluene and methanol in the ratio of 92:8v/v, and the compounds were detected using the vanillin-phosphoric acid reagent by densitometric scanning at 550 nm. The $R_f$ value for STIGMA and LPL was 0.43±0.02and0.55±0.02 with linearity in the range of 100–500 ng/spot and 400-2000 ng/spot, respectively. The mean % recovery was in the range of 93.8–97.2 % for STIGMA and 94.9 –95.6 % for LPL with LOD and LOQ values of 11.74 and 35.58 ng/spot for STIGMA and 90.44 and 274.06 ng/spot for LPL, respectively. Our HPTLC method was accurate and precise for simultaneous estimation of STIGMA and LPL from the bark of *Adenanthera pavonina*, thereby making it effective in quality control investigations of the herbal formulations.

**Keywords:** Adenantherapavonina; HPTLC; Stigmasterol; Lupeol

## INTRODUCTION

Medicinal plantsand plant extracts have been used for ages to treat various diseases and disorders. For this reason, various traditional medicine systems like Ayurveda, Unani, Tibetian, Chinese, African, or Amazonian, based on different cultural models, use medicinal plants for therapeutic effects. The therapeutic potential of these plants results from the presence of secondary metabolites such as alkaloids, steroids, tannins, flavonoids, resins, and fatty acids, some of which also serve as precursors in the synthesis of drugs. However, standardization is significant to ensure the quality of herbal drugs. Identifying the drug, its purity, content, and chemical and biological properties are the deciding factors in controlling the quality of the products. The World Health Organisation (WHO) has developed guidelines for the safe use and quality assurance of medicinal plants. Thus, emphasis is now given to the standardization of medicinal plants and the isolation of active principles.[1] The recent advances in modernized analytical methods have helped develop effective separation strategies of the potent molecules, including HPTLC and RP-HPLC, for the estimation of various chemical compounds present in the herbal formulations

*Adenanthera pavonina* (Family: Fabaceae; Sub-family: Mimosoideae), commonly known as coral wood, redwood, and red bead tree, is a perennial and non-climbing species of leguminous tree distributed over the tropics. Traditionally, the plant *Adenanthera pavonina* is extensively used to treat various diseases.[2,3] The bark extracts of *Adenanthera pavonina* possess bactericidal activity against both the gram-positive and gram-negative bacterial strains.[4] Also, the bark extracts exhibit significant anthelmintic activity, anti-diarrheal activity, and antioxidant properties.[5–7] Research studies have illustrated the anti-inflammatory activity of the

leaf extracts in the mice model and the anti-hypertensive, anti-hyperlipidemic, and anti-hyperglycemic activities of seed extracts in rats.[8–10]

The medicinal properties of *Adenanthera pavonina* are associated with the presence of active phytoconstituents such as non-protein amino acids ($\gamma$-methylene glutamic acid, $\gamma$-methylene glutamine, and traces of $\gamma$-ethylidene glutamic acid), sterols ($\beta$–sitosterol, stigmasterol, and its glucoside), dulcitol and fixed oil (glyceride of palmitic, stearic, arachidic, myristic and lignoceric as saturated fatty acids and oleic and linoleic as unsaturated fatty acids).[11,12]

Stigmasterol and lupeol are significant phytosterols in the plant's bark as they possess anti-inflammatory activities that may be used in multiple therapies.Various chromatographic methods were reported to estimate stigmasterol and lupeolfrom other plants by GC and HPTLC.[7,9] Literature survey revealed no method for simultaneous estimation of stigmasterol and lupeol.The present study was designed to establish a simple, rapid, and optimized HPTLC method to simultaneously estimate lupeol and stigmasterol from the *Adenanthera pavonina* bark extracts.

## MATERIAL AND METHODS

### *Collection of the plant and extraction of its bark*

The fresh bark of the tree of *Adenanthera pavonina* was collected in October 2013 from LM College of Pharmacy, Ahmedabad, Gujarat. The plant was authenticated at the Department of Botany, Gujarat University, Ahmedabad, Gujarat. The bark of *Adenanthera pavonine* was shade dried, pulverized using a mixer grinder, and passed through a 200-mesh sieve to obtain a fine powder. The bark powder (500 g) was weighed, transferred to a round bottom flask (RBF), and dissolved in 500 mL of various solvents like petroleum ether, hexane, chloroform, ethanol, methanol, and ethyl acetate. Finally, petroleum ether was selected as an extraction solvent because it showed maximum extraction and it showed maximum spot on TLC plate. The bark powder was suspended in petroleum ether and was refluxed for 3 h at 85°C. The slurry was filtered through Whatman filter paper no.42. It was further re-extracted (500 mL×4), following the same procedure. All the filtrates were combined, concentrated to 100 mL via distillation, and evaporated to dryness.

### *Reagents and chemicals*

All the solvents,including methanol, toluene, chloroform, petroleum ether, diethyl ether, ethyl acetate, rectified spirit acetone, n-hexane, benzene, ortho-phosphoric acid, and the chemicals silica gel 60–120 #, sodium hydroxide, hydrochloric acid used were of analytical grade and obtained from Finar Chemicals Ltd., Ahmedabad, India.

### *Instrumentation*

The HPTLC system (CAMAG, Switzerland) included CAMAG Linomat IV semi-automatic spotting device, CAMAGTLC Scanner3, CAMAG Twin Trough Chamber (10× 10cm), and CAMAGCATS4 Software. TLC aluminum sheets pre-coated with silica gel 60 $F_{254}$ 20× 20 $cm^2$ with 0.2 mm layer thickness were purchased from E. Merck, Darmstadt, Germany.

### *Chromatographic conditions*

The chromatographic method was developed on pre-coated silica gel 60 $F_{254}$ aluminum sheets (20× 20 $cm^2$) with 0.2 mm layer thickness as stationary phase using different mobile phase compositions. Before applying the sample, the TLC plates were pre-washed with methanol and activated in an oven at 60 °C for 5 minutes. The solutions were applied on a TLC plate in the form of bands of 6 mm width under a stream of nitrogen gas using a CAMAG Linomat IV semiautomatic spotting device with a constant application rate of 10 sec/$\mu$L. The space between the two spots was kept at 5 mm. The plates were developed in a twin trough chamber saturated with the mobile phase for 15 min at 25 ± 2 °C using ascending development technique to 80 mm in length. After development, the plates were air-dried and derivatized with vanillin –phosphoric acid solution. Then the plates were heated for 3 min at 110 °C in a hot air oven, and densitometric scanning was done using CAMAG TLC scanner 3 using CATS 4 software at 550 nm.

### *Preparation of standard solution*

#### *Stock solution of STIGMA*

10 mg of standard STIGMA was weighed accurately and transferred into a 10 mL volumetric flask. STIGMA was dissolved, and volume was made up to the mark using chloroform (1000 $\mu$g/mL). From this stock solution, an aliquot of 1 mL was transferred to a 10 mL volumetric flask, and the volume was made up to the mark using chloroform (100 $\mu$g/mL). This solution was labeled as S1.

#### *Stock solution of LPL*

4 mg of standard LPL was weighed accurately and transferred into a 10 mL volumetric flask. LPL was dissolved, and volume was made up to the mark using chloroform (400 $\mu$g/mL). This solution was labeled as S2.

#### *Working mixed standard solution*

In a series of 10 mL volumetric flasks, aliquots of 1, 2, 3, 4, and 5 mL from S1 and S2 were transferred. The volume was made up to 10 mL using chloroform in each flask to get a concentration of 10, 20, 30, 40, and 50 $\mu$g/mL of STIGMA and 40, 80, 120, 160, and 200 $\mu$g/mL of LPL.

***Preparation of vanillin-phosphoric acid spray reagent***

1 g of vanillin was weighed and transferred to a 100 mL volumetric flask. To this, 50 mL ethanol and 50 mL of orthophosphoric acid were added. A fresh solution was prepared every day of the experiment.

***Development of the HPTLC method and the preparation of the calibration curve***

HPTLC preliminary TLC analysis was performed using n-Hexane: ethyl acetate (90:10v/v) as the mobile phase. The plate was sprayed with respective reagents like 5% conc. sulphuric acid, p-anisaldehyde, vanillin- phosphoric acid, and Liebermann-Burchard to detect steroids or triterpenoids. Several standards of triterpenoids and steroids available in the laboratory were used for comparison with the isolated compound I and II. The $R_f$ value of compound I was found as 0.43 ±0.02, which was similar to LPL, while the $R_f$ value of compound II was found as 0.55 ± 0.02, which was similar to STIGMA (Figure 1).

Calibration was carried out with the dilutions of standards, and triplicate measurement was performed. The linearity of each standard was achieved by plotting peak areas versus standard concentrations (Figure 2 and Figure 3). Peak identification was attained by comparing retention time (RT) and UV absorbance ($\lambda$max).

***Validation of HPTLC method for simultaneous estimation of STIGMA and LPL***

Validation was carried out according to International Conference on Harmonization (ICH) guidelines.[13]

***Linearity and range***

Linearity and range of the method were determined by spotting serial dilution of STIGMA and LPL in the concentration range of 100-500 ng/spot and 400-2000 ng/spot, respectively. There is no reported optimized range as these are not used in any dosage forms. Three sets of each solution were evaluated to obtain a calibration curve. Linearity was obtained by estimating the correlation coefficient, slope, and intercept of the curves.

***Precision***

The present method was validated for intraday and interlay precision. Intraday precision was determined in triplicate with the same method on the same day for three different concentrations of STIGMA (100, 300 and 500 ng/spot) and LPL (400, 1200 and 2000 ng/spot). The interlay precision of the method was verified by performing a similar method on different days under the same set of experimental situations. The repeatability of the sample application and calculation of the peak area for the analyte were articulated in terms of the % RSD.

***Repeatability of measurement of peak area (n=6)***

The repeatability of the peak area was assessed by applying 10 $\mu$L of working mixed standard solution of STIGMA (200 ng/spot) and LPL (800 ng/spot) on the pre-coated plate. The plate was developed using selected chromatographic conditions. The developed spot was scanned six times, peak areas were recorded, and % RSD was calculated.

***Repeatability of sample application (n=6)***

The repeatability of the sample application was assessed by applying six spots of 10 $\mu$L of working mixed standard solution of STIGMA (200 ng/spot) and LPL (800 ng/spot) on the pre-coated plate. The plate was developed using selected chromatographic conditions. The developed spots were scanned, peak areas were recorded, and % RSD was calculated.

***Accuracy***

The accuracy of an analytical method was determined by calculating the recovery of STIGMA and LPL at three different concentration levels (50%, 100%, and 150%) following the standard addition method. The amount of the drug was calculated by employing corresponding linear equations. The average recovery obtained at all three levels was reported as % recovery.

***Limit of detection (LOD) and limit of quantification (LOQ)***

LOD is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified under the stated analytical conditions. In contrast, LOQ is the ability of the analytical method to detect analytes quantitatively in the presence of other compounds. It was calculated as LOD= (3.3 x $\sigma$)/S and LOQ= (10 x $\sigma$)/S, where '$\sigma$' is the standard deviation of the Y-intercept and 'S' is the mean slope of the calibration curve line.

## RESULTS AND DISCUSSION

Among various solvents screened, STIGMA and LPL exhibited the highest peak area in petroleum ether extract, demonstrating that the concentration of the compounds was highest in this solvent. Hence, petroleum ether was selected as a solvent to extract these metabolites from the dried bark. The results are shown in Table 1.

***Characterization of isolated compounds***

The first isolated compound was a white crystalline solid with a melting point in the range of 204–208 °C, which was near the melting point of LPL.[13] The second isolated compound was a white solid with a melting point in the range of 164–167 °C, which was similar to the melting point

**Table 1: Peak areas obtained for STIGMA and LPL in different solvents**

| Solvent used | Average Peak Area of STIGMA | Average Peak Area of LPL |
|---|---|---|
| Petroleum Ether | 4163.43 ± 28.92 | 12726.63 ± 24.34 |
| Hexane | 1673.70 ± 50.31 | 5336.23 ± 56.75 |
| Ethanol | 1751.00 ± 29.85 | 6675.10 ± 47.06 |
| Methanol | 4678.02 ± 39.05 | 4835.57 ± 61.25 |
| Ethyl acetate | 1707.60 ± 17.41 | 6484.73 ± 31.31 |
| Chloroform | 1393.33 ± 15.79 | 4855.73 55.32 |

of stigmasterol reported in the literature.[14] When checked qualitatively using HPTLC with various spraying reagents, vanillin-phosphoric acid spray confirmed the presence of the compounds. Several standard protocols for the steroids and triterpenoids available in the laboratory were used for comparison purposes. The $R_f$ value for the isolated compound I (0.43)was similar to LPL, and that of compound II (0.55) matched with the STIGMA.

### *Development of the HPTLC method*

#### *Selection of chromatographic condition/ optimization of mobile phase*

To select the chromatographic conditions, Silica Gel 60 $F_{254}$pre-coated plates were run in the mobile phases with different compositions (Table 2). The toluene: methanol in the ratio of 92:8 v/v was found satisfactory as it resulted in sharp, symmetric, and well-resolved peaks for STIGMA and LPL at $R_{f\ values}$ of 0.43±0.02 and 0.55±0.02 respectively.

**Table 2: Observations with different mobile phase compositions**

| Trial no. | Mobile phase composition | Observations |
|---|---|---|
| 1 | n-Hexane:Ethyl acetate (90:10 v/v) | Resolution of STIGMA and LPL was not proper |
| 2 | Chloroform: Methanol (90:10 v/v) | Spot of STIGMA moves along with solvent front |
| 3 | Toluene:Ethyl acetate: Methanol (60:20:10 v/v/v) | Tailing was observed |
| 4 | Toluene:Methanol (80:20 v/v) | Undesirable separation of spots |
| 5 | Toluene: Methanol (92:8 v/v) | Proper resolution and $R_f$ value were obtained for both STIGMA and LPL |

### *Calibration curve*

On the silica gel 60 $F_{254}$ pre-coated plate, mixed standard solutions were applied as a 6 mm band in the concentration

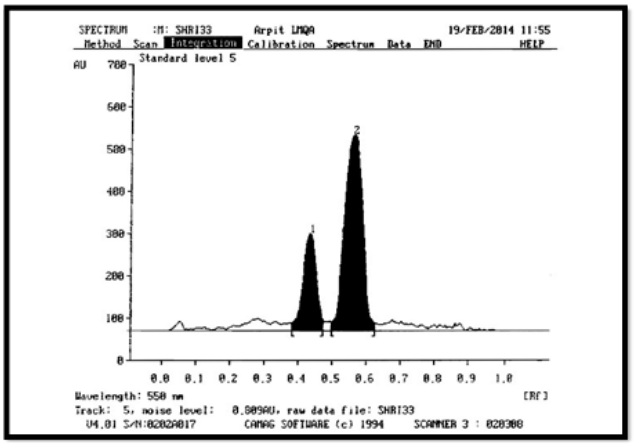


**Fig. 1:** Chromatogram of standard solution of STIGMA and LPL

range of 100–500 ng/spot of STIGMA and 400–2000 ng/spot for LPL. The plate was developed using selected chromatographic conditions, and the respective peak areas were recorded (Figure 1). A graph was plotted using peak area vs. concentration of STIGMA and LPL, respectively. Both graphs were linear with $R^2$ values of 0.9914 and 0.9871 for STIGMA and LPL, respectively

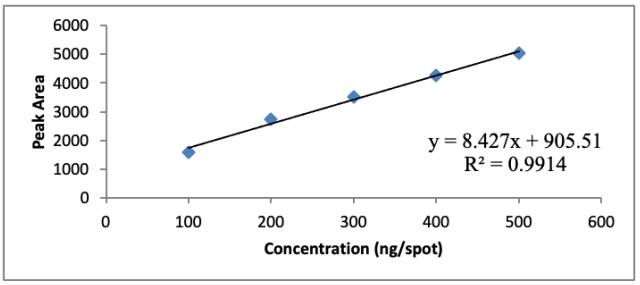


**Fig. 2:** Calibration curve of STIGMA

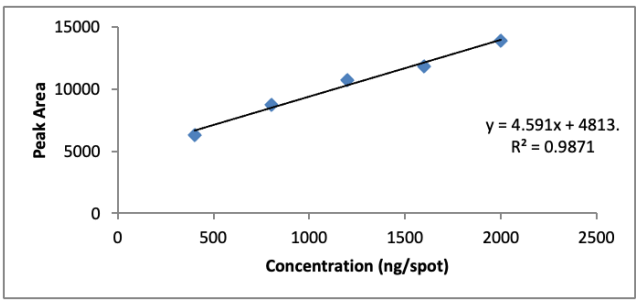


**Fig. 3:** Calibration curve of LPL

### *Sample Analysis*

The sample analysis of the dried extract revealed that STIGMA and LPL were present in the bark in the concentration of 0.0743 % w/w and 0.146 % w/w, respectively.

**Table 4: Intraday and Interday precision**

| Drug | Conc. (ng/spot) | Intra-day Precision | | Inter day Precision | |
|---|---|---|---|---|---|
| | | Peak Area | % RSD | Peak Area | % RSD |
| STIGMA | 100 | 1599.80±12.60 | 0.78 | 1609.63±13.13 | 0.82 |
| | 300 | 3514.80±15.57 | 0.44 | 3495.07±16.56 | 0.47 |
| | 500 | 5072.53±21.62 | 0.42 | 5074.83±39.48 | 0.78 |
| LPL | 400 | 6265.90±38.56 | 0.61 | 6319.7±121.84 | 1.93 |
| | 1200 | 10643.57±46.45 | 0.44 | 10485.17±141.86 | 1.35 |
| | 2000 | 13784.33±91.23 | 0.66 | 13417.37±379.73 | 2.83 |

### *Validation of the developed HPTLC method*

Validation of the developed HPTLC method revealed linearity in the range of 100–500 ng/spot for STIGMA with an $R^2$ value of 0.99 and 400–2000 ng/spot for LPL with an $R^2$ value of 0.9871. The method was precise as % RSD for all the studies was less than 2%. The results are presented in the Table 3 and Table 4 .

**Table 3: Results of repeatability study**

| Sr. no. | Repeatability of Scanner | | Repeatability of Sample Applicator | |
|---|---|---|---|---|
| | Peak Area of STIGMA | Peak Area of LPL | Peak Area of STIGMA | Peak Area of LPL |
| 1 | 2588.5 | 8177.29 | 3115.87 | 8428.78 |
| 2 | 2642.33 | 8037.53 | 3021.91 | 8160.72 |
| 3 | 2620.36 | 8061.41 | 3000.82 | 8251.02 |
| 4 | 2625.81 | 8045.46 | 3043.08 | 8037.31 |
| 5 | 2604.79 | 8144.64 | 3110.79 | 8807.94 |
| 6 | 2657.99 | 7885.79 | 3017.15 | 8300.89 |
| Mean | 2623.29 | 8058.69 | 3051.60 | 8331.11 |
| ± SD | 25.04 | 101.96 | 49.70 | 268.17 |
| % RSD | 0.95 | 1.27 | 1.63 | 3.22 |

The accuracy of the method was further checked using the standard addition method at 50 %, 100 %, and 150 %. Recovery was observed in the range of93.8 to 97.2 % for STIGMA and 94.9 to 95.6 % for LPL. Recovery study data is shown in Table 5 and Table 6. The LOD and LOQ were calculated as11.74 ng/spot and 35.58 ng/spot, respectively, for STIGMA and 90.44 ng/spot and 274.06 ng/spot for LOQ.

## CONCLUSION

The present study presents a unique and validated HPTLC method for the identification and simultaneous quantification of lupeol and stigmasterol *Adenanthera pavonina* bark extracts. The method is goodfor the routine analysis of lupeol and stigmasterol in different crude plants and polyherbal products.

**Table 5: Recovery for STIGMA**

| Amount of stigma in extract (ng/spot) | Amount of Standard Stigma spiked (ng/spot) | Total amount (ng/spot) | Total amount found(ng/spot) Mean±SD (n=3) | Amount of STIGMA Recovered Mean±SD (n=3) | % Recovery Mean±SD (n=3) |
|---|---|---|---|---|---|
| 148.5 | - | 148.5 | 144.3±2.51 | 144.33±2.51 | 97.1±1.21 |
| 148.5 | 75 | 223.5 | 215.2±9.27 | 70.9±5.26 | 94.5±0.75 |
| 148.5 | 150 | 298.5 | 285.1±12.98 | 140.8±6.03 | 93.8±0.65 |
| 148.5 | 225 | 373.5 | 362.9±4.23 | 218.6±8.02 | 97.2±0.94 |

**Table 6: Recovery for LPL**

| Amount of LPL in extract (ng/spot) | Amount of Standard LPL spiked (ng/spot) | Total amount (ng/spot) | Total amount found(ng/spot) Mean±SD (n=3) | Amount of LPL Recovered Mean±SD (n=3) | % Recovery Mean±SD (n=3) |
|---|---|---|---|---|---|
| 292.4 | - | 292.4 | 285.92±5.42 | 285.92±5.42 | 97.78±2.59 |
| 292.4 | 150 | 442.4 | 428.32±7.28 | 142.4±1.18 | 94.9±0.21 |
| 292.4 | 300 | 592.4 | 572.77±10.25 | 286.85±5.45 | 95.6±1.13 |
| 292.4 | 450 | 742.4 | 715.30±6.23 | 429.38±2.95 | 95.4±0.35 |

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