Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading

# Simultaneous HPTLC-UV530 nm analysis and validation of bioactive lupeol and stigmasterol in *Hygrophila auriculata* (K. Schum) Heine

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ARTICLE INFO

*Article history:*
Received 2 May 2012
Received in revised form 13 May 2012
Accepted 31 August 2012
Available online 28 August 2012

*Keywords:*
*Hygrophila auriculata*
HPTLC-UV
Lupeol
Stigmasterol
Validation

ABSTRACT

**Objective:** To analyse the two marker compounds lupeol (LP) and stigmasterol (ST) from methanolic extract of *Hygrophila auriculata* (*H. auriculata*). **Methods:** Separation was achieved on aluminium plates precoated with silica gel 60$F_{254}$ with toluene-methanol-formic acid (7.0: 2.7: 0.3 v/v/v) as mobile phase. **Results:** Densitometric analysis was performed at 530 nm in the reflectance mode. Compact bands for LP and ST were obtained at $R_f$ 0.52 ± 0.02 and 0.28 ± 0.05. Linearity ($r^2$=0.998 5 and 0.993 7), limit of detection (45 and 18 ng/band) limit of quantification (135 and 54 ng/band), recovery (98.2%-99.7% and 97.2%-99.6%), and precision (<2.18 and 1.91) were satisfactory for LP and ST respectively. Linearity range for LP and ST were 100-1 000 and 50-500 ng/band and the contents estimated as (0.19±0.1)% and (0.47±0.1)% w/w respectively. **Conclusion:** The method demonstrated efficient analysis testing of LP and ST in samples; therefore it can be used for routine analysis.

## 1. Introduction

*Hygrophila auriculata* (*H. auriculata*) (*K. Schum*) Heine (HA), a generally occurring wild herb belonging to Acanthaceae family has been advocated for the treatment of variety of diseases including most commonly diabetes and dysentery[1-3]. As per our tradition, roots, seeds, and aerial parts of the plant has been used in the treatment of jaundice, hepatic obstruction, rheumatism, inflammation, urinary infection, gout, malaria and impotence[4]. The plant has been reported to contain flavonoids (apigenin 7-O-glucuronide, apigenin 7-O-glucoside)[5], alkaloids (asteracanthine and asteracanthicine)[6], aliphatic esters (25-oxo-hentricontyl acetate, methyl-8-hexyltetracosanoate)[7], minerals (Fe, Cu, Co)[8], sterols (stimagsterol)[9], triterpenes (lupeol, hentricotane, betulin, luteolin, luteolin 7-O-rutinosides)[7,10] and essential oils[6]. Earlier scientific investigation showed that the crude extract of HA has anti-nociceptive[11], antitumor[12,13], antibacterial[14,15], antioxidant[16,17], hepatoprotective[18-20], hypoglycemic[21], haematinic[22], diuretic[23] anabolic and androgenic activities[24]. Nowadays, HPTLC has become a routine analytical technique due to its reliability in quantitation of analytes at nanolevel estimation and cost effectiveness[25-28]. HPTLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plant extracts. A little information is only available regarding analytical methods for the qualitative and/or quantitative estimation of lupeol (1R, 3aR, 5aR, 5bR, 7aR, 9S, 11aR, 11bR, 13aR, 13bR,)-3a,5b, 8,8, 11a hexamethyl-1-prop-1-ene-2-yl-1, 2, 3, 4, 5,6, 7, 7a, 9,10, 11, 11b, 12, 13, 13a, 13b-hexadecahydrocyclopenta [a] chrysene-9-ol) and stigmasterol (3S, 8S, 9S, 10R, 13R, 14S, 17R)-17-[(E2R, 5S)-5-ethyl-6-methyl hept-3-en-2-yl] - 10, 13- dimethyl-2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17- dodecahydro-1H- cyclopenta [a] phenanthren-3-ol) (Figure 1 A, B & 2). A capillary gas chromatographic method has been developed for the qualitative analysis of sterols and triterpenes[29], however, the HPTLC chromatographic fractionation of the main constituent's sterols and triterpenes has also been published[30]. Earlier estimations have been done both for LP and ST in other plants either individual or simultaneous, by using hyphenated techniques like HPTLC[31-33], LC-MS/MS[34] and gas chromatography[35]. However, pertaining to our knowledge there is no any hyphenated HPTLC technique available anywhere else for simultaneous estimation of LP and ST in HA extract. So, the attempt has been made to accept this challenge towards development and validation of LP and ST simultaneously by such a hyphenated technology like HPTLC-UV for the betterment of herbal quality standards.

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## 2. Materials and methods

### 2.1. Plant material and chemicals

*H. auriculata* fresh plant were collected from the field area of Saharsa, Bihar, India in the month of January 2009; and the specimens (voucher no: SHC 55/01/2009 ) were authenticated by Dr. Anjani kumar Sinha (taxonomist), Department of Botany MLT Saharsa College, Bihar. Standard stigmasterol (Purity: 97% w/w) and lupeol (purity: 99% w/w) were purchased from Natural Remedies Pvt. Ltd, Bangalore, India. All the solvents used were of chromatography grade and other chemicals used were of analytical reagent (AR) grade. Precoated silica gel 60 $F_{254}$ HPTLC plates were purchased from E. Merck, Germany.

### 2.2. Preparation of standard and quality control (QC) samples

Stock solutions of LP and ST (10 mg/mL) were prepared in methanol, and by appropriate dilution standard solutions were prepared in the concentration range of 0.1 to 1.0 mg/mL. For calibration, LP standard solution (1-10 μL) was applied to a HPTLC plate to furnish amounts in the range 100-1 000 ng/band, however ST standard solution (0.5-5 μL) was applied to furnish amounts in the range 50-500 ng/band. Peak area and amounts applied were treated by linear least-squares regression. Each amount was applied six times. QC samples as low, medium and high at concentration level of 200, 400 and 800 ng/band were taken for LP and 100, 200 and 400 were considered for ST to carry out validation of the method.

### 2.3. Chromatography

Chromatography was performed, as described previously [25-28] on 20 cm × 10 cm aluminum Lichrosphere HPTLC plates precoated with 200-μm layers of silica gel 60$F_{254}$ (E. Merck, Darmstadt, Germany). Samples were applied as bands 6 mm wide and 10 mm apart by means of Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100 μL syringe. The constant application rate was 160 nL/s. Linear ascending development with toluene-methanol-formic acid (7.0: 2.7: 0.3 v/v/v) as mobile phase was performed in a 20 cm × 10 cm twin-trough glass chamber (Camag) previously saturated with mobile phase for 15 min at room temperature (25±2) ℃ and relative humidity 60%±5%.

The development distance was 8 cm (development time 10 min) and 20 mL mobile phase was used. The plates were dried at room temperature in air and derivatized with anisaldehyde-sulphuric acid reagent and warmed (at 75 ℃ for 5 min) to identify compact bands. Densitometric analysis was performed at 530 nm in reflectance mode with a Camag TLC scanner III operated by WinCATS software (Version 1.2.0). The slit dimensions were 5 mm × 0.45 mm and the scanning speed of 20 mm/s.

### 2.4. HPTLC-UV530nm fingerprinting and image analysis

The plants were air-dried and pulverized. 500 g of the powdered material were packed in muslin cloth and subjected to soxhlet extractor for continuous hot extraction with methanol for 72 h. Thereafter methanolic extracts of HA were filtered through Whatman paper no. 42 and the resultant filtrates were concentrated under reduced pressure and finally vacuum dried. The yield of the methanolic extract was 13.2% w/w. The protocol for preparing sample solutions was optimized for high quality fingerprinting and also to extract the marker compounds efficiently. Since the marker compounds were soluble in methanol, therefore methanol was used for extraction.

The fingerprinting of HA extracts were executed by spotting 10 μL of suitably diluted sample solution of the methanolic extract on a HPTLC plate. Each amount was applied six times. Peak area and amounts applied were treated by linear least-squares regression. The plates were developed and scanned as same discussed above. The peak areas were recorded and the amount of stigmasterol and lupeol was calculated using the calibration curve.

### 2.5. Method validation

Validation of the developed method has been carried out as per ICH guidelines for linearity, range, precision, accuracy, limits of detection (LOD) and quantification (LOQ), and recovery.

#### 2.5.1. Precision and accuracy

Precision (inter and intraday) and accuracy of the assay were evaluated by performing replicate analyses ($n$=6) of QC samples at low, medium and high QC levels of 200, 400 and 800 ng/band for LP and 100, 200 and 400 ng/band for ST, respectively. Inter-day precision and accuracy were determined by repeating the intra-day assay on three different days. Precision was expressed as the coefficient of variation (CV, %) of measured concentrations for each calibration level whereas accuracy was expressed as percentage recovery [(Drug found/drug applied) × 100].

#### 2.5.2. Robustness

Robustness was studied in triplicate at 400 ng/band by making small changes to mobile phase composition, mobile phase volume, and duration of mobile phase saturation and activation of TLC plates, the effect on the results were examined by calculation of RSD (%) and SE of peak areas. Mobile phases prepared from toluene-methanol-formic acid (7.0: 2.7: 0.3 v/v/v) in different proportions (6.5: 3.2: 0.3, v/v/v, 6.8: 2.9: 0.3, v/v/v, 7.2: 2.5: 0.3, v/v/v, and 7.0:2.7:0.3, v/v/v) keeping the volume formic acid constant were used for chromatography. Mobile phase volume and duration of saturation investigated were (20±2) mL (18, 20, and 22 mL) and (20±10) min (10, 20, and 30 min), respectively. The plates were activated at (60±5) ℃ for 2, 5, and 7 min before

chromatography.

### 2.5.3. Sensitivity

To estimate the limits of detection (LOD) and quantification (LOQ), blank methanol was applied six times and the standard deviation (σ) of the analytical response was determined. The LOD was expressed as 3 σ/slope of the calibration plot and LOQ was expressed as 10 σ/slope of the calibration plot.

### 2.5.4. Recovery studies

Recovery was studied by applying the method to drug samples to which known amounts of marker corresponding to 50%, 100%, and 150% of the LP or ST had been added. Each level was analyzed in triplicates. This was to check the recovery of LP or ST at different levels in the extracts. Recovery of the markers at different levels in the samples was determined.

## 3. Results

### 3.1. Chromatography

Chromatogram were developed for both LP and ST under chamber saturation conditions using toluene-methanol-formic acid (7.0: 2.7: 0.3 v/v/v) as mobile phase or solvent system (Figure 1B&C). The same mobile phase has been also employed for the separation of HA methanolic extracts (Figure 1D). The optimized saturation time was found to be 10 min. UV spectra measured for the spots showed maximum absorbance at about 530 nm therefore UV densitometry analysis was performed at 530 nm in the reflectance mode as HPTLC-UV530nm. Compact bands as sharp, symmetrical and with high resolution were obtained at $R_F$ (0.52±0.02) and (0.28±0.05) for LP and ST respectively (Figure 2).

As far as we are aware, there is no any HPTLC-UV method reported to quantify LP and ST simultaneously in HA herb or extracts. Therefore we have attempted to develop and validate a cost effective simple and sober UV hyphenated HPTLC technique to quantify bioactive marker components in this herb. LP and ST were well resolved at $R_F$ 0.52 and 0.28 respectively (Figure 1A, 1B & 1C) from HA methanolic extract sample in the solvent system as same used in case of standards.

The plates were visualized at two different wavelengths 254, 366 and 530 nm as the compounds were found to absorb at variable spectrum range. In addition, this helped in the generating a better fingerprint data whereby species could be well differentiated on enhanced visual identification of individual compounds. The method developed here was found to be quite selective with good baseline resolution of each compound (Figure 1A).

The identity of the bands of compounds 1-9 in the sample extracts was confirmed by overlaying their UV absorption spectra with those of the standards at 530 nm (Table 1).

### 3.3. Calibration

**Table 1**
TLC fingerprints of HA extracts at 530 nm.

| S. No. | $R_F$ value | Color of the band |
|---|---|---|
| 1 | 0.13 | Light blue band |
| 2 | 0.22 | Blue |
| 3 | 0.28 (ST) | Intense blue |
| 4 | 0.39 | Blue |
| 5 | 0.52 (LP) | Green |
| 6 | 0.59 | Purple |
| 7 | 0.64 | Light blue |
| 8 | 0.69 | Light blue |
| 9 | 0.78 | Red |

Linearity of compounds (LP and ST) was validated by the linear regression equation and correlation coefficient. The six-point calibration curves for LP and ST were found to be linear in the range of 100-1 000 ng/band and 50-500 ng/band. Regression equation and correlation coefficient for the reference compound were: Y = 0.005 9X (0.999 4) for LP, and Y = 0.013X-0.037 for ST (0.994 1), which revealed a good linearity response for developed method and are presented in Table 2. The mean values (± sd) of the slope were 0.005 9

**Table 2**
$R_F$, linear regression data for the calibration curve and sensitivity parameter for LP and ST.

| Parameter | $R_F$ | Linearity range (ng/band) | Regression equation | $r^2$ | Slope±sd | Intercept±sd | Standard error of slope | Standard error of intercept | LOD | LOQ |
|---|---|---|---|---|---|---|---|---|---|---|
| LP | 0.52 | 100-1 000 | Y=0.005 9X+0 | 0.999 4 | 0.005 9±0.000 8 | nil | 0.001 1 | na* | 45 | 75 |
| ST | 0.28 | 50-500 | Y=0.013X-0.037 | 0.994 1 | 0.013±0.006 | 0.037±0.004 | 0.003 | 0.014 | 18 | 49 |

*not available (na).

**Table 3**
Precision and accuracy of the method (intraday batch/interday batch).

| Group | Nominal concentrationa | Obtained[a,b] | Precision[c] | Accuracy[d] |
|---|---|---|---|---|
| Lupeol | 200 | 198.3/196.2 | 1.80/2.18 | 99.1/98.1 |
| | 400 | 396.8/392.8 | 1.75/1.86 | 99.2/98.2 |
| | 800 | 801.4/798.3 | 1.53/1.70 | 100.2/99.7 |
| Stigmasterol | 100 | 97.5/95.47 | 1.73/1.91 | 97.5/95.7 |
| | 200 | 198.6/196.9 | 1.84/1.78 | 99.3/98.4 |
| | 400 | 402.2/396.7 | 1.37/1.55 | 100.5/99.2 |

[a]Concentration in ng/band; [b]Mean from six determinations ($n$=6); [c]Precision as coefficient of variation (CV, %)=[(standard deviation)/(concentration ound)]×100; [d]Accuracy(%)=[concentration found)/(nominal concentration)]×100.



**Table 4**
Robustness of the method.

| Optimisation condition | LP | | ST | |
|---|---|---|---|---|
| | SD | %RSD | SD | %RSD |
| Mobile phase (toluene-methanol-formic acid; proportions (6.5: 3.2: 0.3, v/v/v, 6.8: 2.9: 0.3, v/v/v, 7.2: 2.5: 0.3, v/v/v, and 7.0:2.7:0.3, v/v/v) | 1.63 | 1.52 | 1.59 | 1.35 |
| Mobile-phase volume (18, 20, and 22 mL) | 1.38 | 1.27 | 1.12 | 0.98 |
| Duration of saturation (10, 20, and 30 min) | 1.92 | 1.83 | 1.07 | 0.91 |
| Activation of TLC plates (2, 5, and 7 min) | 1.19 | 1.08 | 1.43 | 1.22 |

**Table 5**
Recovery studies of LP and ST.

| Group | Concentration added to analyte (%) | Theoretical (ng) | Added (ng) | Detected (ng) | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|---|
| Lupeol | 50 | 400 | 200 | 589.3 | 98.2 | 1.92 |
| | 100 | 400 | 400 | 793.6 | 99.2 | 1.51 |
| | 150 | 400 | 600 | 996.8 | 99.7 | 1.49 |
| Stigmasterol | 50 | 200 | 100 | 291.5 | 97.2 | 1.14 |
| | 100 | 200 | 200 | 395.2 | 98.8 | 1.89 |
| | 150 | 200 | 300 | 497.8 | 99.6 | 1.17 |

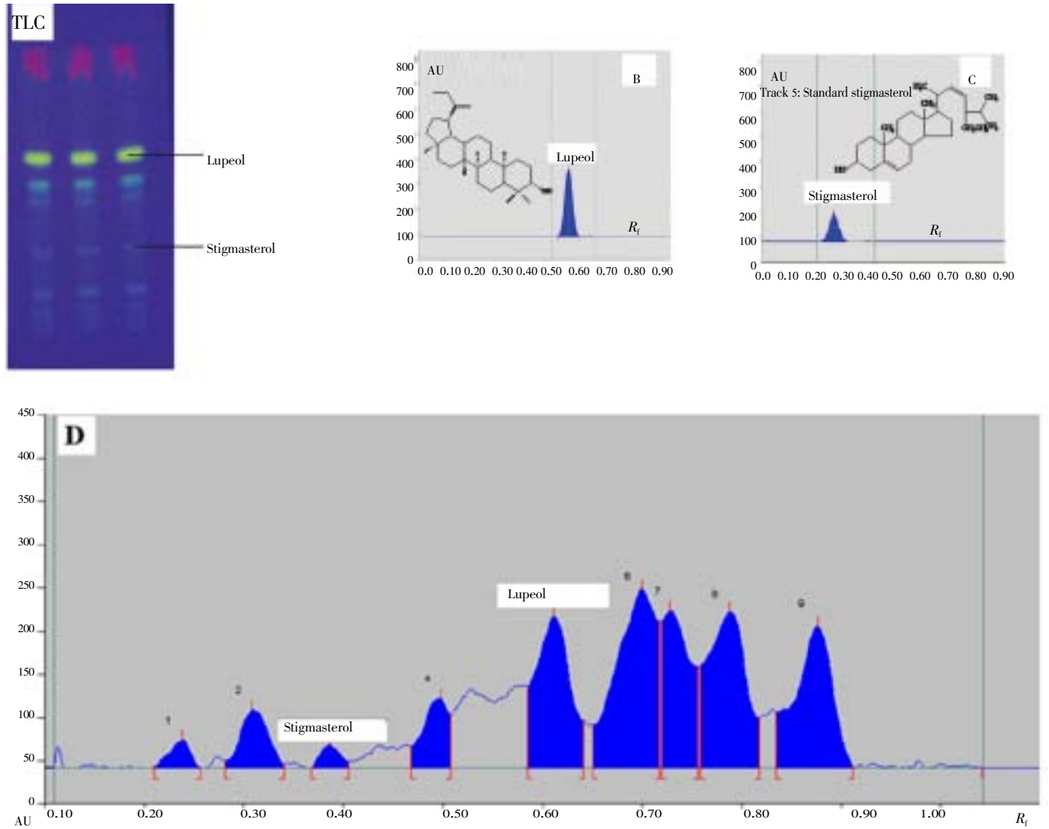


**Figure 1.** (A) TLC profile of *H. auriculata* (K. Schum) Heine methanolic extract after derivatization at 530 nm; Spot indicates stigmasterol and lupeol, respectively, (B) HPTLC chromatogram of standard lupeol structure at $R_F$ 0.52, (C) HPTLC chromatogram of standard stigmasterol with structure at $R_F$ 0.28 and (D) HPTLC chromatogram of methanolic extract of *H. auriculata* (K. Schum) Heine scanned at 530 nm [peak 1-11; LP (0.52) and ST (0.28)].

±0.0008 and 0.013±0.006 and intercept were zero and 0.037 ±0.004 respectively for LP and ST. No significant difference was observed in the slopes of standard plots (ANOVA, $P > 0.05$).

### 3.4. Method validation

#### 3.4.1. Precision and accuracy

Table 3 presents intra-day and inter-day precision (as coefficient of variation, % CV) and accuracy of the assay for LP and ST at three QC levels (200, 400 and 800 ng/band). Intra-day precisions ($n = 6$) for LP and ST were ⩽ 1.80% and ⩽ 1.84%, however the inter-day precisions were ⩽ 2.18% and ⩽ 1.91% respectively, which demonstrated the good precision of proposed method. Intra-day accuracy for LP and ST were 99.1%-100.2% and 97.5%-100.5%, however inter-day accuracy for LP and ST were 98.1%-99.7% and 95.7%-99.2% respectively. These values are within the acceptable range, so the method was accurate, reliable, and reproducible.

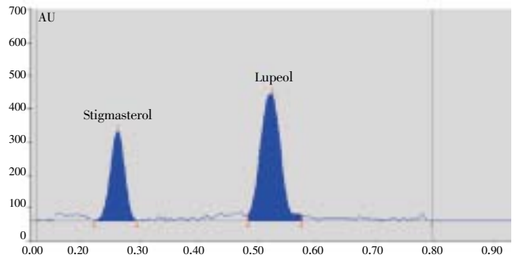


**Figure 2.** Chromatogram of LP and ST simultaneously determined in *H. auriculata* (K. Schum) Heine methanolic extract by using toluene-methanol-formic acid (7.0: 2.7: 0.3 v/v/v) as solvent system scanned at 530 nm [LP (0.52) and ST (0.28)].

#### 3.4.2. Robustness

The SD and % RSD was calculated for LP and ST. The low value of SD and % RSD obtained after introducing small deliberate changes in the method indicated that the method was robust (Table 4).

#### 3.4.3. Sensitivity

LOD values for LP and ST were 45 and 18 ng/band respectively; however LOQ values were 135 and 54 ng/band respectively (Table 2), indicating adequate assay sensitivity. The LOD and LOQ were determined from the slope of the lowest part of the calibration plot. This indicated that the proposed method exhibits a good sensitivity for the quantification of above compounds.

#### 3.4.4. Recovery studies

Good recoveries were obtained by the fortification of the sample at three QC levels for LP and ST. It is evident from the results that the percent recoveries for both markers after sample processing and applying were in the range of 98.2%-99.7% (LP) and 97.2%-99.6% (ST) for as shown in Table 5.

### 3.5. HPTLC-UV530nm analysis of bioactive LP and ST in HA extract

The content of LP and ST was estimated in the HA methanolic extract by the proposed method and the results obtained are summarized in Table 6. The percentage of LP and ST obtained in the extract were 0.19 and 0.47 respectively with RSD. It is for the first time, a simple, accurate and rapid HPTLC method has been developed for the simultaneous quantification of bioactive compounds in HA.

## 4. Discussion

The presented study clearly gave evidence of the simultaneous bioactive quantitative of LP and ST in HA extracts. The developed hyphenated HPTLC method for the simultaneous quantification of above marker compounds is simple, precise, specific, sensitive, and accurate. Further, this method can be effectively used for routine quality control of herbal materials as well as formulations containing any or both of these compounds.

## Conflict of interest

We declare that we have no conflict of interest.

## Acknowledgement

The author and co-authors would like to acknowledge our Honorable Vice Chancellor, Integral Universitry, Lucknow, for providing necessary facilities in university premises for this research. Author, is also thankful to Dr. K.F.H. Nazeer Ahamed, Assistant Professor, Deptt. of Pharmacology, Vel's college of Pharmacy, Chennai, for his unprecedented support and encouragement. We also would like to extent our sincere thanks to Mr. Md. Zaheen Hassan Ansari Research Scholars at Faculty of Pharmacy, Jamia Hamdard, New Delhi, for critically reading the manuscript and providing the valuable suggestions.

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