Journal of Coastal Life Medicine

Journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.2.2014J23
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Phytochemical investigation and simultaneous estimation of bioactive lupeol and stigmasterol in *Abutilon indicum* by validated HPTLC method

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PEER REVIEW

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Comments

The paper describes a simple method to identify two biologically active compounds widespread in plants. The method is simple and above all inexpensive. The accuracy of HPTLC method is verified.
Details on Page 400

ABSTRACT

Objective: To perform a simultaneous quantitative estimation of two biologically active triterpenoid compounds lupeol and a steroid compound, stigmasterol, in *Abutilon indicum* (*A. indicum*) using high-performance thin-layer chromatography (HPTLC).

Methods: TLC aluminum plates precoated with silica-gel 60 F254 (20 cm×10 cm) were used with a mobile phase of toluene-methanol-formic acid (7.0:2.7:0.3, v/v/v) and densitometric determination of these compounds was carried out at 530 nm in reflectance/absorbance mode.

Results: Compact bands for lupeol and stigmasterol were obtained at R_f 0.52±0.02 and 0.28±0.05. The 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 lupeol and stigmasterol respectively. Linearity range for lupeol and stigmasterol were 100-1000 (r^2=0.999 4) and 50-500 ng/band (r^2=0.994 1) and the contents were estimated as (0.59±0.10)% and (0.83±0.10)% w/w respectively. The total phenolic, flavonoid, proanthocyanidin, alkaloidal and saponin contents of methanolic extract of *A. indicum* were also measured in this work. According to International Conference on Harmonization (ICH) guidelines, the method was validated for linearity, precision, accuracy, and recovery, limit of detection, limit of quantification, specificity, and robustness.

Conclusions: The HPTLC method was found to be reproducible, accurate, and precise and could detect these two compounds at nanogram level from the *A. indicum*.

KEYWORDS

Abutilon indicum (Linn) Sweet, HPTLC-UV_{530}, Lupeol, Stigmasterol, Validation

1. Introduction

Abutilon indicum L. (Malvaceae) (*A. indicum*), known commonly as "Thuthi", is distributed throughout the hotter parts of India[1]. The juice from its leaves has been used to formulate into an ointment for quick ulcer healing[2,3]. Its extract is also used in relieving thirst; in treating bronchitis, diarrhea, gonorrhea, inflammation of the bladder; and in reducing fever. The leaves are effective in ulcer, for the treatment of diabetes, diuretic infection and gingivitis[4,5]. Fomentation of plant materials are used to relieve body pain. The decoction of the leaf is used in toothache, tender gums and internally for inflammation of bladder. In some places, juice from the leaves in combination with the liquid extract of *Allium cepa* is used to treat jaundice, and in cases of hepatic disorders[6,7]. The leaves and seeds are

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Article history:
Received 4 Dec 2013
Received in revised form 8 Jan, 2nd revised form 13 Feb, 3rd revised form 8 Mar 2014
Accepted 30 Mar 2014
Available online 28 May 2014

crushed with water to form paste which is applied to penis to cure syphilis[8-10]. In Siddha system of medicine, it is used as a remedy for jaundice, piles, ulcer and leprosy[11]. The plant was reported to contain many active and inactive compounds[12,13]. Previous phytochemical investigations showed that it contains saponins, flavonoids, alkaloids, and essential oils[14]. In another investigation gallic acid, β-sitosterol, β-amyrin, eudesmol, eugenol, geraniol, and caryophyllene were reported[13,15]. Subramanian and Nair 1972 and Sharma and Ahmad 1989 reported separately the isolation of gossypetin-7-glucoside, cyanidin-3-rutinoside, and two sesquiterpene lactones named alantolactone and isoalantolactone, in addition to gossypetin-8-glucoside[5,15,16].

Nowadays, high performance thin layer chromatography (HPTLC) is becoming a routine analytical technique due to its advantages of low operating cost; high sample throughput and need for minimum sample clean up. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis[17-21]. HPTLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plant extracts. A little information is 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 1A and B). The above two compounds are reported to be present in many medicinal plants and have shown to have significant biological activities. Lupeol was reported to show anti-inflammatory[22], antioxidant[23], antiplasmodial[24], thyroid inhibitory[25], antiperoxidative[26], hypoglycemic and hypocholesteromic activity[27]. Stigmasterol is reported to show antioxidant[28], anti-inflammatory[29], analgesic and anthelmintic[30], hepatoprotective and anticancer activity[31-35]. Thin-layer chromatography (TLC) and HPTLC methods are commonly applied for the identification, assay and testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics, nutriments)[36]. These flexible and cost-effective techniques present the advantage of the simultaneous processing of standards and samples with versatile detection possibilities, including a great variety of post-chromatographic derivatization reagents. A capillary gas chromatographic method has been developed for the qualitative analysis of sterols and triterpenes[37]. However, the HPTLC chromatographic fractionation of the main constituent's sterols and triterpenes has also been published[38]. Earlier estimations have been done both for lupeol and stigmasterol in other plants either individual or simultaneous, by using hyphenated techniques like HPTLC[39-42], liquid chromatography-mass spectrometry/mass spectrometry and gas chromatography[43]. However, as far as our knowledge is concerned, there is not any hyphenated HPTLC technique available in any other report for simultaneous estimation of lupeol and stigmasterol in methanolic extract of *A. indicum*. So, the attempt has been made to accept this challenge towards development and validation of lupeol and stigmasterol simultaneously by such a hyphenated technology like HPTLC-UV for the betterment of herbal quality standards.

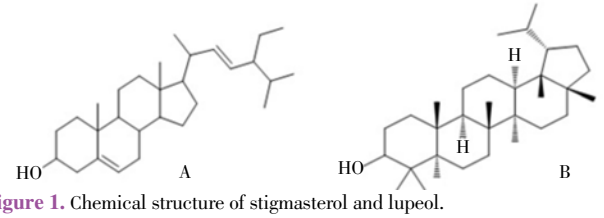

Figure 1. Chemical structure of stigmasterol and lupeol.

2. Materials and methods

2.1. Plant material and chemicals

The fresh plant of *A. indicum* (Linn) Sweet were collected from the field area of Saharsa (Bihar), India in the month of April 2009, and the specimens (voucher No. SHC 57/05/2009) 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 grade. Precoated silica gel 60 F254 HPTLC plates were purchased from E. Merck, Germany.

2.2. Determinations of total phenolics, flavonoid, proanthocyanidin, alkalaoid and saponin contents

The total phenolic content was determined according to the method described by Singleton *et al*[44]. A suitable aliquot of the methanolic extract was placed in test tubes and made up to 1 mL with distilled water. Then, 0.5 mL Folin-Ciocalteu reagent (1:1 with water) and 2.5 mL sodium carbonate solution (20%) were added sequentially to each tube. Then, the tubes were vortexed for 2 min, kept in the dark for 40 min and the absorbance was recorded at 725 nm. The amount of total phenolics was calculated as gallic acid equivalents/mg of extract.

Flavonoid contents were measured using a modified colorimetric method by Jia *et al*[45]. Extract solution (0.25 mL, 1 mg/mL) was added to a test tube containing 1.25 mL of distilled water. Sodium nitrite solution (5%, 0.075 mL) was added to the mixture and maintained for 5 min. Then, 0.15 mL of 10% aluminum chloride was added. After 6 min, 0.5 mL of

1 mol/L sodium hydroxide was finally added. The mixture was diluted with 0.275 mL of distilled water. The absorbance of the mixture at 510 nm was measured immediately in comparison to a standard curve prepared by quercetin. The flavonoid contents were expressed as mg quercetin equivalent/g dry basis.

Contents of proanthocyanidins were determined by the procedure of Sun *et al*[46]. Five hundred microliters of methanolic extract solution was mixed with 3 mL of 4% vanillin-methanol solution and 1.5 mL hydrochloric acid. The mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm, while the final result was expressed as mg catechin equivalent/g dry basis.

Contents of alkaloid were determined by the procedure of Harborne[47]. Five grams of the sample was weighed into a 250 mL beaker and 200 mL of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Contents of saponin were determined by the procedure of Obadoni and Ochuko 2001[48]. The sample was ground and 20 g of each were put into a conical flask and 100 mL of 20% aqueous ethanol was added. The sample was heated over a hot water bath for 4 h with continuous stirring at about 55 °C. The mixture was filtered and the residue was re-extracted with another 200 mL 20% ethanol. The combined extracts were reduced to 40 mL over water bath at about 90 °C. The concentrate was transferred into a 250 mL separatory funnel and 20 mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. A volume of 60 mL of *n*-butanol was added. The combined *n*-butanol extracts were washed twice with 10 mL of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage.

2.3. Chromatography

Chromatography was performed, as described previously on 20 cm×10 cm aluminum LiChrosphere HPTLC plates precoated with 200-μm layers of silica gel 60F254 (E. Merck, Darmstadt, Germany)[17-21]. 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) °C 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 °C 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 5.00 mm×0.45 mm and the scanning speed of 20 mm/s.

2.4. Preparation of standard and quality control (QC) samples

Stock solutions of lupeol and stigmasterol (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, lupeol standard solution (1-10 μL) was applied to a HPTLC plate to furnish amounts in the range 100-1000 ng/band, however stigmasterol standard solution (0.5-5.0 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 lupeol and 100, 200 and 400 were considered for stigmasterol to carry out validation of the method.

2.5. HPTLC-$UV_{530\ nm}$ fingerprinting and image analysis

The whole plant of *A. indicum* were air-dried and pulverized. Five hundred grams of the powdered material was packed in muslin cloth and subjected to Soxhlet extractor for continuous hot extraction with methanol for 72 h. Thereafter methanolic extracts of *A. indicum* 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 17.3% 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 methanolic extracts of *A. indicum* 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 International Conference on Harmonization (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 lupeol and 100, 200 and 400 ng/band for stigmasterol, 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 relative standard deviation (RSD) (%) and SE of peak areas. Mobile phases prepared from toluene-methanol-formic acid 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) °C for 2, 5, and 7 min before chromatography.

2.5.3. Sensitivity

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

2.5.5. 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 lupeol or stigmasterol had been added. Each level was analyzed in triplicates. This was to check the recovery of lupeol or stigmasterol at different levels in the extracts. Recovery of the markers at different levels in the samples was determined.

3. Results

3.1. Determinations of total phenolics, flavonoid, proanthocyanidin, alkalaoid and saponin contents

The content of total phenolics, flavonoids, proanthocyanidin, alkaloid and saponin was determined in the methanolic extract of *A. indicum* by the proposed method and the results obtained are summarized in Table 1.

Table 1
Determinations of total phenolics, flavonoid, proanthocyanidin, alkalaoid and saponin contents in *A. indicum*.

Quantitative parameters	*A. indicum*
Phenolics (μg/mL)	98.50±0.56
Flavonoids (μg/mL)	97.38±1.53
Proanthocyanidin (μg/mL)	17.71±1.52
Alkaloids (% w/w)	2.45±0.39
Saponin (% w/w)	1.62±0.19

3.2. Chromatography

Chromatogram were developed for both lupeol and stigmasterol under chamber saturation conditions using toluene-methanol-formic acid (7.0:2.7:0.3, v/v/v) as mobile phase or solvent system (Figures 2 and 3). The same mobile phase has been also employed for the separation of methanolic extracts of *A. indicum* (Figure 4). 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 densitometric analysis was performed at 530 nm in the reflectance mode as HPTLC-$UV_{530\ nm}$. Compact bands as sharp, symmetrical and with high resolution were obtained at R_f (0.52±0.02) and (0.28±0.05) for lupeol and stigmasterol respectively (Figure 5). As far as we are aware, there is not any HPTLC-UV method reported to quantify lupeol and stigmasterol simultaneously in *A. indicum* 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. lupeol and stigmasterol were well resolved at R_f 0.52 and 0.28 respectively (Figures 2 and 3) from *A. indicum* 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 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 (Figures 2 and 3). The identity of the bands of compounds 1-11 in the sample extracts was confirmed by overlaying their UV absorption spectra with those of the standards at 530 nm (Table 2).

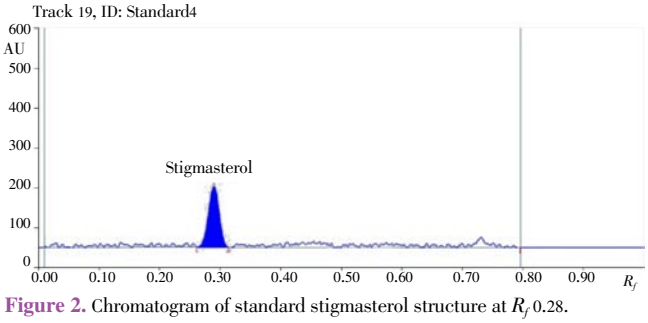

Figure 2. Chromatogram of standard stigmasterol structure at R_f 0.28.

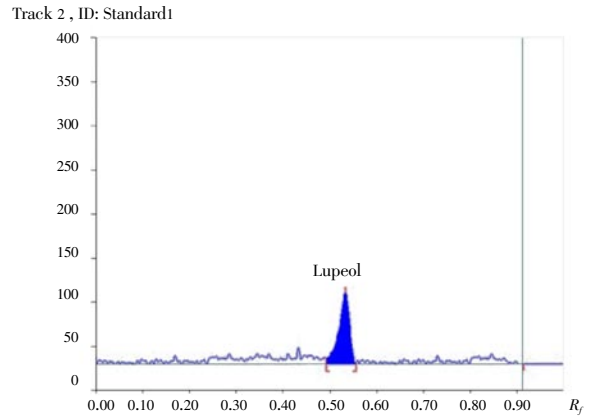

Figure 3. Chromatogram of standard lupeol with structure at R_f 0.52.

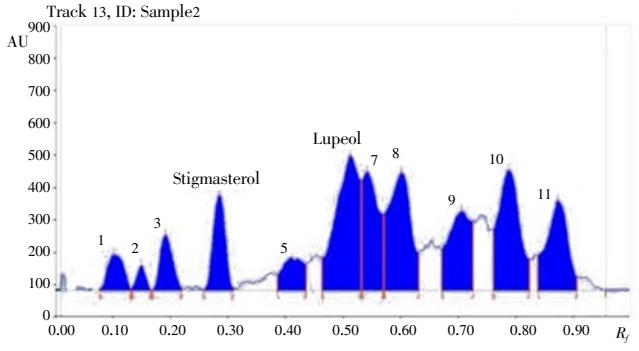

Figure 4. Chromatogram of methanolic extract of *A. indicum* scanned at 530 nm. Peak 1-11; Stigmasterol: 0.28; Lupeol: 0.52.

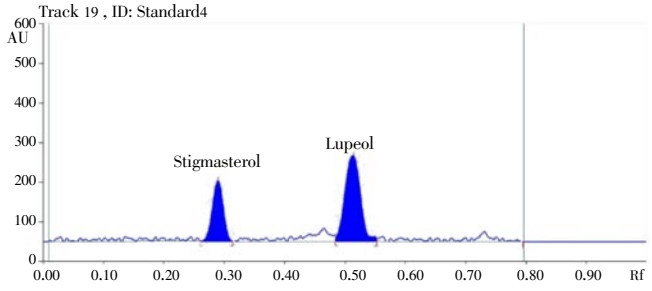

Figure 5. Chromatogram of lupeol and stigmasterol simultaneously determined in *A. indicum* methanolic extract by using toluene-methanol-formic acid (7.0:2.7:0.3 v/v/v) as solvent system scanned at 530 nm. Stigmasterol: 0.28; Lupeol: 0.52.

3.3. Calibration

Linearity of compounds (lupeol and stigmasterol) was validated by the linear regression equation and correlation coefficient. The six-point calibration curves for lupeol and stigmasterol were found to be linear in the range of 100-1000 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 lupeol, and Y=0.013x-0.037 for stigmasterol (0.994 1), which revealed a good linearity response for developed method and are presented in Table 3. The mean values (±SD) of the slope were 0.005 9±0.000 8 and 0.013±0.006 and intercept were zero and 0.037±0.004 respectively for lupeol and stigmasterol. No significant difference was observed in the slopes of standard plots (ANOVA, $P>0.05$).

Table 2

TLC fingerprints of methanolic extract of *A. indicum* at 530 nm.

S. No.	R_f value
1	0.11
2	0.15
3	0.19
4	0.28 (stigmasterol)
5	0.42
6	0.52
7	0.55 (lupeol)
8	0.59
9	0.72
10	0.79
11	0.87

Table 3

R_f, linear regression data for the calibration curve and sensitivity parameter for lupeol and stigmasterol.

Parameter	Lupeol	Stigmasterol
R_f	0.52	0.28
Linearity range (ng/band)	100-1 000	50-500
Regression equation	Y=0.005 9x+0	Y=0.013x-0.037
Correlation coefficient (r^2)	0.999 4	0.994 1
Slope±SD	0.005 9±0.000 8	0.013±0.006
Intercept±SD	0	0.037±0.004
Standard error of slope	0.001 1	0.003
Standard error of intercept	Not available	0.014
LOD	45	18
LOQ	135	54

3.4. Method validation

3.4.1. Precision and accuracy

Table 4 presents intra-day and inter-day precision (as coefficient of variation, %CV) and accuracy of the assay

Table 4

Precision and accuracy of the method.

Intra/inter-day	Lupeol				Stigmasterol			
	Nominal concentration[a]	Obtained[a,b]	Precision[c]	%Recovery[d]	Nominal concentration[a]	Obtained [a,b]	Precision[c]	%Recovery[d]
Intraday batch	200	198.3	1.80	99.1	100	97.5	1.73	97.5
	400	396.8	1.75	99.2	200	198.6	1.84	99.3
	800	801.4	1.53	100.1	400	402.2	1.37	100.5
Interday batch	200	196.2	2.18	98.1	100	95.7	1.91	95.7
	400	392.8	1.86	98.2	200	196.9	1.78	98.4
	800	798.3	1.70	99.7	400	396.7	1.55	99.2

[a]: Concentration in ng band^{-1}; [b]: Mean from six determinations (n=6); [c]: Precision as coefficient of variation (CV, %) = [(standard deviation)/(concentration found)]×100; [d]: Accuracy (%) = [concentration found)/(nominal concentration)]×100.

for lupeol and stigmasterol at three QC levels (200, 400 and 800 ng/band). Intra-day precisions (n=6) for lupeol and stigmasterol were ≤1.80% and ≤1.84%, however the inter-day precisions were ≤2.18% and ≤1.78% respectively, which demonstrated the good precision of proposed method. Intra-day accuracy for lupeol and stigmasterol were 99.1%-100.1% and 97.5%-100.5%, however inter-day accuracy for lupeol and stigmasterol 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.

3.4.2. Robustness

The SD and %RSD was calculated for lupeol and stigmasterol. The low value of SD and %RSD obtained after introducing small deliberate changes in the method indicated that the method was robust (Table 5).

Table 5

Robustness of the method.

Optimisation condition	Lupeol		Stigmasterol	
	SD	%RSD	SD	%RSD
Mobile phase (toluene-methanol-formic acid; (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

3.4.3. Sensitivity

LOD values for lupeol and stigmasterol were 45 and 18 ng/band respectively; however LOQ values were 135 and 54 ng/band respectively (Table 3), 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 lupeol and stigmasterol. 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% (lupeol) and 97.2%-99.6% (stigmasterol) as shown in Table 6.

Table 6

Recovery studies of lupeol and stigmasterol.

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

3.4.5. HPTLC-$UV_{530\ nm}$ analysis of bioactive lupeol and stigmasterol in methanolic extract of A. indicum

The content of lupeol and stigmasterol was estimated in the methanolic extract of *A. indicum* by the proposed method. The content of of lupeol and stigmasterol obtained in the extract were 0.59 and 0.83 ng/spot respectively with RSD of 1.14% and 1.76% respectively. It is for the first time, a simple, accurate and rapid HPTLC method has been developed for the simultaneous quantification of two bioactive compounds in *A. indicum*.

4. Discussion

HPTLC is a simple, rapid and accurate method for analyzing plant material. HPTLC fingerprint has better resolution and estimation of active constituents is done with reasonable accuracy in a shorter time. The HPTLC method can be used for phytochemical profiling of plants and quantification of compounds present in plants. With increasing demand for herbal products as medicines and cosmetics there is an urgent need for standardization of plant products[49]. Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to traditional system of medicine throughout the world. The optimized chromatographic finger print is not only an alternative analytical tool for authentication, but also an approach to express the various patterns of chemical ingredients distributed in the herbal drugs and to preserve such "database" for further multifaceal sustainable studies[50]. HPTLC finger print analysis has become the most of its simplicity and reliability. It can serve as a tool for identification, authentication, qualitative, quantitative analysis and quality control of herbal drug[51]. The presented study clearly gave evidence of the simultaneous bioactive quantitative of lupeol and stigmasterol in methanolic extracts of *A. indicum*. 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 statement

We declare that we have no conflict of interest.

Acknowledgements

The corresponding author, Mr. Md. Sarfaraj Hussain with all contributed authors would like to acknowledge our Honorable Vice Chancellor, for providing necessary facilities in university premises for this research. We also would like to extent our sincere thanks to Dr. Saeed Ahmad, Associate Professor, Department of Pharmacognosy & Phytochemistry, Faculty of Pharmacy, Hamdard University, New Delhi, for his unprecedented support and encouragement and providing

HPTLC facilities for this research.

Comments

Background

Leaves of *A. indicum* were used in Indian folk medicine to treat many diseases. The extract shows antinflammatory, antiulcer and hypoglycemic effect. Literature data report that the plant contains a lot of active principles, as saponins, flavonoids, alkaloids, essential oil and sesquiterpene lactones.

Research frontiers

The authors are interested in lupeol and stigmasterol, two compounds present in many medicinal plants, whose biological activity is well known.

Related reports

Phytochemical analysis were performed to identify and quantify active principles responsible for the biological activity of the medicinal plant.

Innovations and breakthroughs

The authors performed simultaneous quantitative evaluation of lupeol and stigmasterol by means of a simple and inexpensive method. The method was validated for precision, accuracy, recovery and the results are interesting. Therefore they determined the content of phenols, flavonoids, proanthocyanidins, alkaloids and saponins.

Applications

The phytochemical analysis fully justify the use of *A. indicum* in traditional medicine. The determination of lupeol and stigmasterol by means of HPTLC can be applied to other medicinal plants.

Peer review

The paper describes a simple method to identify two biologically active compounds widespread in plants. The method is simple and above all inexpensive. The accuracy of HPTLC method is verified.

References

[1] Chopra RN, Chopra IC, Handa KL, Kapoor LD. *Indigenous drugs of India*. Calcutta, India: Dhur UN and Sons Pvt. Ltd.; 1958, p. 456, 561.

[2] Gaind KN, Chopra KS. Phytochemical investigation of *Abutilon indicum*. *Planta Med* 1976; **30**: 174-188.

[3] Nadkarni AK. *Indian materia medica*. 3rd ed. Bombay, India: Popular Book Depot; 1954, p. 6.

[4] Subramanian SS, Nair AGR. Flavonoids of four *Malvaceous* plants. *Phytochem* 1972; **11**: 1518-1519.

[5] Porchezhian E, Ansari SH. Effect of liquid extract from fresh *Abutilon indicum* leaves and *Allium cepa* bulbs on paracetamol and carbontetrachloride induced hepatotoxicity. *Pharmazie* 2000; **55**: 702-703.

[6] Porchezhian E, Ansari SH. Hepatoprotective activity of *Abutilon indicum* on experimental liver damage in rats. *Phytomedicine* 2005; **12**: 62-64.

[7] Dhanalakshmi S, Lakshmanan KK, Subramanaian MS. Pharmacognostical and phytochemical studies of *Abutilan* L. *J Res Educ Indian Med* 1990; 21-25.

[8] Jain A, Katewa SS, Chaudhary BL, Galav P. Folk herbal medicines used in birth control and sexual diseases by tribals of southern Rajasthan India. *J Ethnopharmcol* 2004; **90**: 171-177.

[9] Jain A, Katewa SS, Chaudhary BL, Galav P. Medicinal plant diversity of sitamata wildlife sanctuary, Rajasthan, India. *J Ethnopharmcol* 2005; **102**: 143-157.

[10] Yoganarasimhan SW. *Medicinal plants of India*. Vol. 2. Bangalore, India: Cyber Media; 2000, p. 10-11.

[11] Mehta BK, Neogi R, Kotra S. Antimicrobial activity of *Abutilon indicum*. *Fitoterapia* 1997; **68**: 273-274.

[12] Dennis TJ, Kumar KA. Chemical examination of the roots of *Abutilon indicum* Linn. *J Oil Technol Assoc Ind* 1987; **15**: 82-83.

[13] Sinha SKP, Dogra JVV. Asurvey of the plants of Bhagalpur and Santthal Pargana for saponin, flavonoids and alkaloids. *Pharm Biol* 1985; **23**: 77-86.

[14] Sharma PV, Ahmad ZA. Two sesquiterpene lactones from *Abutilon indicum*. *Phytochem* 1989; **28**: 3525.

[15] Yasmin S, Kashmiri MA, Ahmad I, Adnan A, Ahmad M. Chemical constituents of *Abutilon indicum* Linn. *Pharm Biol* 2008; **46**: 673-676.

[16] Hussain MS, Sheeba F, Ali M. Simultaneous HPTLC-$UV_{530\ nm}$ analysis and validation of bioactive lupeol and stigmasterol in *Hygrophila auriculata* (K. Schum) Heine. *Asian Pac J Trop Biomed* 2012; **2**(Suppl 2): S612-S617.

[17] Hussain MS, Sheeba F, Ali M. Phytochemical investigation and hyphenated chromatographic analysis of bioactive lupeol and stigmasterol in *Sespadula* by validated high-performance thin layer chromatography method. *J Nat Pharm* 2012; **3**(1): 46-53.

[18] Hussain MS, Sheeba F, Ali M. Hyphenated chromatographic analysis of bioactive gallic acid and quercetin in *Hygrophila auriculata* (K. Schum) Heine growing wildly in marshy places in India by validated HPTLC method. *Asian Pac J Trop Biomed* 2012; **2**(Suppl 2): S477-S483.

[19] Hussain MS, Sheeba F, Ali M. Validation of the method for the simultaneous estimation of bioactive marker gallic acid and quercetin in *Abutilon indicum* by HPTLC. *Asian Pac J Trop Dis* 2012; **2**(Suppl 1): S76-S83.

[20] Faiyazuddin Md, Rauf A, Ahmad N, Ahmad S, Iqbal Z, Talegaonkar S, et al. A validated HPTLC method for determination of terbutaline sulfate in biological samples: application to pharmacokinetic study. *Saudi Pharm J* 2011; **19**: 185-191.

[21] Geetha T, Varalakshmi P. Anti-inflammatory activity of lupeol and lupeol linoleate in rats. *J Ethnopharmacol* 2001; **76**(1): 77-80.

[22] Shirwaikar A, Shetty M, Bommu P. Effect of lupeol isolated from *Crataeva nurvala* Buch.-Ham. stem bark extract against free

radical induced nephrotoxicity in rats. *Ind J Exp Biol* 2004; **42**(7): 686-690.

[23] Ziegler HL, Franzyk H, Sairafianpour M, Tabatabai M, Tehrani MD, Bagherzadeh K, et al. Erythrocyte membrane modifying agents and the inhibition of *Plasmodium falciparum* growth: structure-activity relationships for betulinic acid analogues. *Bioorg Med Chem* 2004; **12**(1): 119-127.

[24] Panda S, Jafri M, Kar A, Meheta BK. Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from *Butea monosperma*. *Fitoterapia* 2009; **80**: 123-126.

[25] Jamaluddin F, Mohamed S, Lajis MN. Hypoglycaemic effect of *Parkia speciosa* seeds due to the synergistic action of β-sitosterol and stigmasterol. *Food Chem* 1994; **49**: 339-345.

[26] Battaab AK, Xuab G, Hondac A, Miyazakic T, Salena G. Stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the rat. *Metabolism* 2006; **55**: 292-299.

[27] Vivancos M, Moreno JJ. Beta-sitosterol modulates antioxidant enzyme response in RAW 264.7 macrophages. *Free Radic Biol Med* 2005; **39**(1): 91-97.

[28] Delporte C, Backhouse N, Erazo S, Negrete R, Vidal P, Silva X, et al. Analgesic-anti-inflammatory properties of *Proustia pyrifolia*. *J Ethnopharmacol* 2005; **99**(1): 119-124.

[29] McAnuff MA, Harding WW, Omoruyi FO, Jacobs H, Morrison EY, Asemota HN. Hypoglycemic effects of steroidal sapogenins isolated from Jamaican bitter yam, *Dioscorea polygonoides*. *Food Chem Toxicol* 2005; **43**(11): 1667-1672.

[30] Sunitha S, Nagaraj M, Varalakshmi P. Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defence system in cadmium-induced hepatotoxicity in rats. *Fitoterapia* 2001; **72**: 516-523.

[31] Chaturvedi PK, Bhui K, Shukla Y. Lupeol: connotations for chemoprevention. *Cancer Lett* 2008; **263**: 1-13.

[32] Saleem M, Alam A, Arifin S, Shah MS, Ahmed B, Sultana S. Lupeol, a triterpene, inhibits early responses of tumor promotion induced by benzoyl peroxide in murine skin. *Pharmacol Res* 2001; **43**: 127-134.

[33] Nigam N, Prasad S, George J, Shukla Y. Lupeol induces p53 and cyclin-B-mediated G2/M arrest and targets apoptosis through activation of caspase in mouse skin. *Biochem Biophys Res Commun* 2009; **381**: 253-258.

[34] Saleem M. Lupeol, a novel anti-inflammatory and anticancer dietary triterpene. *Cancer Lett* 2009; **285**: 109-115.

[35] Biringanine G, Chiarelli MT, Faes M, Duez P. A validation protocol for the HPTLC standardization of herbal products: application to the determination of acteoside in leaves of *Plantago palmata* Hook. *Talanta* 2006; **69**: 418-424.

[36] Kpoviéssi DSS, Gbaguidia F, Gbénou J, Accrombessia G, Moudachiroua M, Rozetd E, et al. Validation of a method for the determination of sterols and triterpenes in the aerial part of *Justicia anselliana* (Nees) T. Anders by capillary gas chromatography. *J Pharm Biomed Anal* 2008; **48**: 1127-1135.

[37] Haliński P, Szafranek J, Szafranek BM, Gołębiowski M, Stepnowski P. Chromatographic fractionation and analysis of the main components of eggplant (*Solanum melongena* L.) leaf cuticular waxes. *Acta Chromatographica* 2009; **21**: 127-137.

[38] Saboo S, Tapadiya G, Khadabadi S. Morpho-anatomy, physicochemical and phytochemical standardization with HPTLC fingerprinting of aerial parts of *Rivea hypocrateriformis*. *Asian Pac J Trop Biomed* 2012; **2**(Suppl 2): S689-S694.

[39] Anandjiwala S, Srinivasa H, Rajani M. Isolation and TLC densitometric quantification of gallicin, gallic acid, lupeol and β-sitosterol from *Bergia suffruticosa*, a hitherto unexplored plant. *Chromatographia* 2007; **66**: 725-734.

[40] Padashetty SA, Mishra SH. An HPTLC method for the evaluation of two medicinal plants commercially available in the Indian market under the common trade name Brahmadandi. *Chromatographia* 2007; **66**: 447-449.

[41] Nazneen MDB, Wesely EG, Johnson M. High performance thin layer chromatography profile studies on the alkaloids of *Albizia lebbeck*. *Asian Pac J Trop Biomed* 2012; **2**(Suppl 1): S1-S6.

[42] Kamran A, Mujeeb M, Altaf A, Mohd A, Nasar M, Deepak S. Validated HPTLC analysis method for quantification of variability in content of curcumin in *Curcuma longa* L (turmeric) collected from different geographical region of India. *Asian Pac J Tropical Biomed* 2012; **2**(Suppl 1): S584-S588.

[43] Safeena S, Suhail A, Ahmad S. Development of HPTLC method and its validation for the estimation of curcuminoids from polyherbal mouth ulcer gel formulation. *IOSR J Pharm* 2013; **3**(1): 29-34.

[44] Sunita S, Menon S, Neelam S, Bhavesh T. Simultaneous estimation of three terpenoids *Carissa carandas* using validated high performance thin layer chromatography. *Int J Green Pharm* 2012; **6**(3): 241-247.

[45] Manjula K, Rajendran K, Eevera T, Kumaran S. Quantitative estimation of lupeol and stigmasterol in *Costus igneus* by high-performance thin-layer chromatography. *J Liq Chromatogr Relat Technol* 2013; **36**(12): 197-212.

[46] Pawar RK, Shivani S, Singh KC, Sharma Rajeev KV. Physico-chemical standardization and development HPTLC method for the determination of Andrographonin in Kalmgeh Navyas Loha-an Ayurvedic formulation. *J Curr Pharm Res* 2011; **2**(1): 43-48.

[47] Mauji R, Abdin MZ, Khan MA, Prabhakar J. *HPTLC fingerprint analysis: a quality control of authentication of herbal phytochemicals*. Berlin Heidelberg,Germany: Springer Verlag; 2011, p. 105.

[48] Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteau reagent. *Methods Enzymol* 1999; **299**: 152-178.

[49] Chang C, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal* 2002; **10**: 178-182.

[50] Sun BS, Ricardo-Da-Silva JM, Spranger MI. Critical factors of vanillin assay for catechins and proanthocyanidins. *J Agric Food Chem* 1998; **46**: 4267-4274.

[51] Obdoni BO, Ochuko PO. Phytochemical studies and comparative efficacy of the crude extracts of some homostatic plants in Edo and Delta States of Nigeria. *Global J Pure Appl Sci* 2001; **8**: 203-208.