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Original Article

High-Performance Thin Layer Chromatography Methods for Estimation of Chemical Markers in *Stachytarpheta indica* Vahl.

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ABSTRACT

Background: *Stachytarpheta indica* Vahl. (Verbenaceae family), commonly known as Indian Snakeweed, is a valuable medicinal plant. A systematic phytochemical analysis of bioactives of this plant is lacking till date. **Objectives:** The present study is aimed to develop validated High-Performance Thin-Layer Chromatography (HPTLC) methods for quantification of 6 marker compounds, *β*-sitosterol, lupeol, ursolic acid, apigenin, caffeic acid and verbascoside in *S. indica* plant. **Materials and Methods:** Normal phase chromatography was used to develop mobile phase systems that could aid in the identification and quantification of the phytochemicals in the entire plant. Several enhancements were made to the basic method of thin-layer chromatography to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurement. The methods were validated as per the International Council for Harmonization (ICH) guidelines. **Results:** The linear regression analysis data for the calibration plots showed a good linear relationship (r2=0.9873-0.9979) in the concentration range of 1-5 µg for *β*-sitosterol, lupeol and ursolic acid; 0.5-5 µg for apigenin, 1-3 µg for caffeic acid; and 3-8 µg per spot for verbascoside with respect to area. The average recoveries for phytoconstituents were 98.32 to 98.97%, indicating the good reproducibility. Verbascoside 1.3598±0.014% w/w was present at high concentration and ursolic acid 0.0027±0.0007% w/w was present at low concentration in the whole plant powder. **Conclusion:** The validated TLC-densitometric methods were simple, precise, accurate, specific and can be used for the routine quality control of raw materials of *S. indica*.

Keywords: Apigenin, Caffeic acid, High-performance thin-layer chromatography, *Stachytarpheta indica*, Ursolic acid, Verbascoside.

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INTRODUCTION

Stachytarpheta indica Vahl. (Family: Verbenaceae), is a prominent pantropical species indigenous to tropical America, Eastern Africa and South-East Asia.[1-3] The plant is widely cultivated as an ornamental plant in different parts of the world and is also well-known for its curative effects in respiratory disorders, ulcers, diarrhea and dropsy. It is reported to be useful in the treatment of fever, rheumatic inflammation, intestinal worms and venereal diseases.[4,5] Collated scientific literature aiming search on phytochemicals present in the plant linked presence of iridoid glycosides (ipolamiide and 6 *β*-hydroxyipolamiide), sterols and triterpenoids (*β*-sitosterol, lupeol and stigmasterol), triterpenic acid (ursolic acid) and flavonoids (luteolin, hispidulin, apigenin and scutellarein) as major categories.[6-11] The use has attracted various health claims and ensuant pursuit on pharmacological swot performed to examine the possible beneficial health effects such as antimicrobial, insecticidal,[12] anti-diarrheal,[13] anthelmintic[14] anti-nociceptive, anti-inflammatory[15] and antihypertensive activities.[16,17] However, there are no documented references on the quantitative phytochemical analysis of *S. indica* plant. Thus, in the light of this information, we propose to establish high-performance thin-layer chromatography methods to demarcate the phytoconstituents of *S. indica*. Preliminary Thin-Layer Chromatographic (TLC) analysis using a linear ascending technique showed the occurrence of the secondary metabolites like sterols, triterpene acids and flavonoids in various extracts of *S. indica*. The sample extracts of the plant, when run along with the standards, indicated presence of *β*-sitosterol, lupeol, ursolic acid, apigenin, caffeic acid and verbascoside in various extracts. Different chromatographic parameters were optimized for optimum separation of the referred components. *β*-sitosterol and lupeol estimated by simultaneous HPTLC method. The developed method showed acceptable validation parameters according to ICH guidelines

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and thus can be employed for the quantification of these marker compounds in *S. indica* efficiently.

MATERIALS AND METHODS

Plant Material

Fresh whole plants of *S. indica* were collected in full bloom from a local nursery farm at Ahmedabad, Gujarat, India. The plant was identified and authenticated by the taxonomist of Gujarat University, Gujarat, India. Voucher specimen has been deposited at the department of the authors. The fresh plants were cut, properly dried, powdered and passed through 60# sieve. The powdered drug was stored in airtight containers at room temperature.

Chemicals and solvents

Petroleum ether (60-80°C), ethyl acetate, 2N hydrochloric acid, Polyvinylpyrrolidone (PVP), toluene, sodium carbonate, ethanol, methanol, dioxane, acetic acid, formic acid, anisaldehyde and sulphuric acid (E. Merck, Mumbai, India) of analytical grade were used. All the reference standards *β*-sitosterol, lupeol, ursolic acid, apigenin, caffeic acid and verbascoside (purity 99.9%) were purchased from Sigma-Aldrich Chemicals (India).

Extraction of plant material

135 g of accurately weighed powdered plant material was defatted with petroleum ether (5×1.0 L) under reflux condition. The petroleum ether extract was evaporated to dryness under vacuum to yield Extract-A. After drying the defatted plant material, 95% ethanolic (5×600 mL) extract was prepared from it. The ethanolic extract was then filtered, concentrated and the tannins were precipitated by adding 200 mL saturated solution of PVP. After removing the precipitates, the extract was dried under vacuum to yield Extract-B. The dried Extract B was dissolved in 200 mL aqueous ethanol (10%) and hydrolyzed under reflux using 1:1 mixture of 2N hydrochloric acid and toluene at 100°C for 2 hr. The hydrolyzed extract, after neutralizing with sodium carbonate solution (10%), was extracted thrice with ethyl acetate (3×50 mL) and dried to yield Extract-C.

Quantification of marker compounds using HPTLC method

Estimations of *β*-sitosterol, lupeol and ursolic acid in Extract-A, verbascoside in Extract-B and apigenin and caffeic acid in Extract-C were done of *S. indica* by the newly developed and validated HPTLC methods.

Instrument and Chromatographic Conditions

The instrument used was Camag Linomat 5 applicator (Muttenz, Switzerland), Camag micro-syringe (100 µL), CAMAG twin-trough chamber and a Camag TLC scanner 3 S/N 130319 with winCATS software and a Camag TLC visualizer for the photo documentation of the plates. The following requirements were taken into consideration: slit dimensions, 5×0.42 mm; scanning speed, 20 mm/s; spraying rate, 10 s/µL; data resolution, 100 mm/step. The chromatographic plates used were aluminum plates precoated with silica gel $60F_{254}$ (20×20 cm, 0.25 mm) of E. Merck, Darmstadt, Germany. Experimental conditions: temperature, 25±2°C; relative humidity, 40%; slit dimension: 5.00 mm×0.45 mm, scanning speed: 10 mm s^{-1}. For *β*-sitosterol, lupeol, ursolic acid solvent system used was toluene: methanol (9.6: 0.4, v/v), visualization agent anisaldehyde-sulfuric acid reagent followed by heating temperature 110°C for 1 min., detection wavelength 545 nm using deuterium lamp; Mobile phases developed for apigenin and caffeic acid were toluene: dioxane: acetic acid (9.5: 2.5: 0.5, v/v/v) and toluene: ethylacetate: formic acid (5: 4: 1, v/v/v) respectively and the plates were detected 366 nm. Ethyl acetate: methanol: water (20: 3.3: 1.7, v/v/v) was used for verbascoside and detection at 254 nm.

Preparation of test solutions

5 mg of accurately weighed test extract A were dissolved in 1 mL methanol and spotted with 5 and 15 µL volumes for estimation of *β*-sitosterol, lupeol and ursolic acid. 5 mg of Extract-B was dissolved in 1 mL methanol and spotted for 10 µL on the TLC plate for estimation of verbascoside. 1 mg of Extract-C was dissolved in 1 mL methanol were separately dissolved in 1 mL methanol and then spotted for 10 and 25 µL volumes for estimation of caffeic acid and apigenin respectively. Each test extracts were sonicated in an ultrasonic bath (Model: TRANS-O-SONIC, Frequency: 50 Hz) for 5 min for complete solubilisation before spotting.

Preparation of standard solutions

5 mg of each reference standard, *β*-sitosterol, lupeol, ursolic acid, apigenin, caffeic acid and verbascoside, was dissolved in 5 mL of methanol separately by sonicating in an ultrasonic bath for 5 min to yield stock solutions (1 µg/µL).

Development of calibration curves

Graded concentrations of standard solutions, 1-5 µL for *β*-sitosterol, ursolic acid and lupeol, 0.5-5 µL for apigenin, 1-3 µL for caffeic acid and 3 to 8 µL for verbascoside, were applied on precoated TLC silica gel $60F_{254}$ plates in triplicates using Camag Linomat V automatic spotter (band width 6 mm and distance between tracks 12 mm). The plates were developed in a twin-trough chamber (20×10 cm) up to a distance of 8 cm using 10 mL of mobile phase at 25±2°C temperature and 40% relative humidity. The mobile phases used were toluene: methanol (9.6: 0.4, v/v) for *β*-sitosterol, lupeol and ursolic acid; toluene: dioxane: acetic acid (9.5: 2.5: 0.5, v/v/v) for apigenin; toluene: ethylacetate: formic acid (5: 4: 1, v/v/v) for caffeic acid and ethyl acetate: methanol: water (20: 3.3: 5, v/v/v) for verbascoside. The plates were scanned at 545 nm for *β*-sitosterol, lupeol and ursolic acid after derivatization with anisaldehyde-sulphuric acid reagent.

Densitometric scanning was carried out at 254 nm for verbascoside and apigenin and 366 nm for caffeic acid. The calibration curves were obtained by plotting area vs. concentration of each peak corresponding to the respective spot.

High-performance thin-layer chromatography analysis

Preliminary chromatographic analysis, carried out by TLC using silica gel G $60F_{254}$ as the stationary phase, suggested the presence of *β*-sitosterol, lupeol and ursolic acid in Extract-A, verbascoside in Extract-B and apigenin and caffeic acid in Extract-C. These marker compounds were quantified using the respective extracts by HPTLC methods using silica gel G $60F_{254}$ as stationary phase and the optimized mobile phases as mentioned earlier. During each repetition of the methods, a fresh mobile phase was prepared. After derivatization with anisaldehyde-sulphuric acid reagent, the plates were scanned at 545 nm for *β*-sitosterol, lupeol and ursolic acid, at 254 nm for verbascoside and apigenin and at 366 nm for caffeic acid. The amount of each compound was calculated from the peak area corresponding to the respective spots.

Validation of HPTLC methods

The developed methods were validated based on the linearity, precision and repeatability, specificity, accuracy, Limit of Detection (LOD) and Limit of Quantification (LOQ) as per the ICH guidelines.[18]

Linearity

Under the experimental conditions described previously, linear relationships were studied from the calibration curves derived by plotting the concentrations of test extracts against respective peak

Table 1: **Amounts of phyto-constituents in *S. indica* plant.**

Phyto-constituent	β-Sitosterol	Lupeol	Ursolic acid	Apigenin	Caffeic acid	Verbascoside
gm % w/w	0.1223+0.0016	0.0746+0.0006	0.0027+0.0007	0.0766+0.0013	0.0445+0.00428	1.3598+0.0140

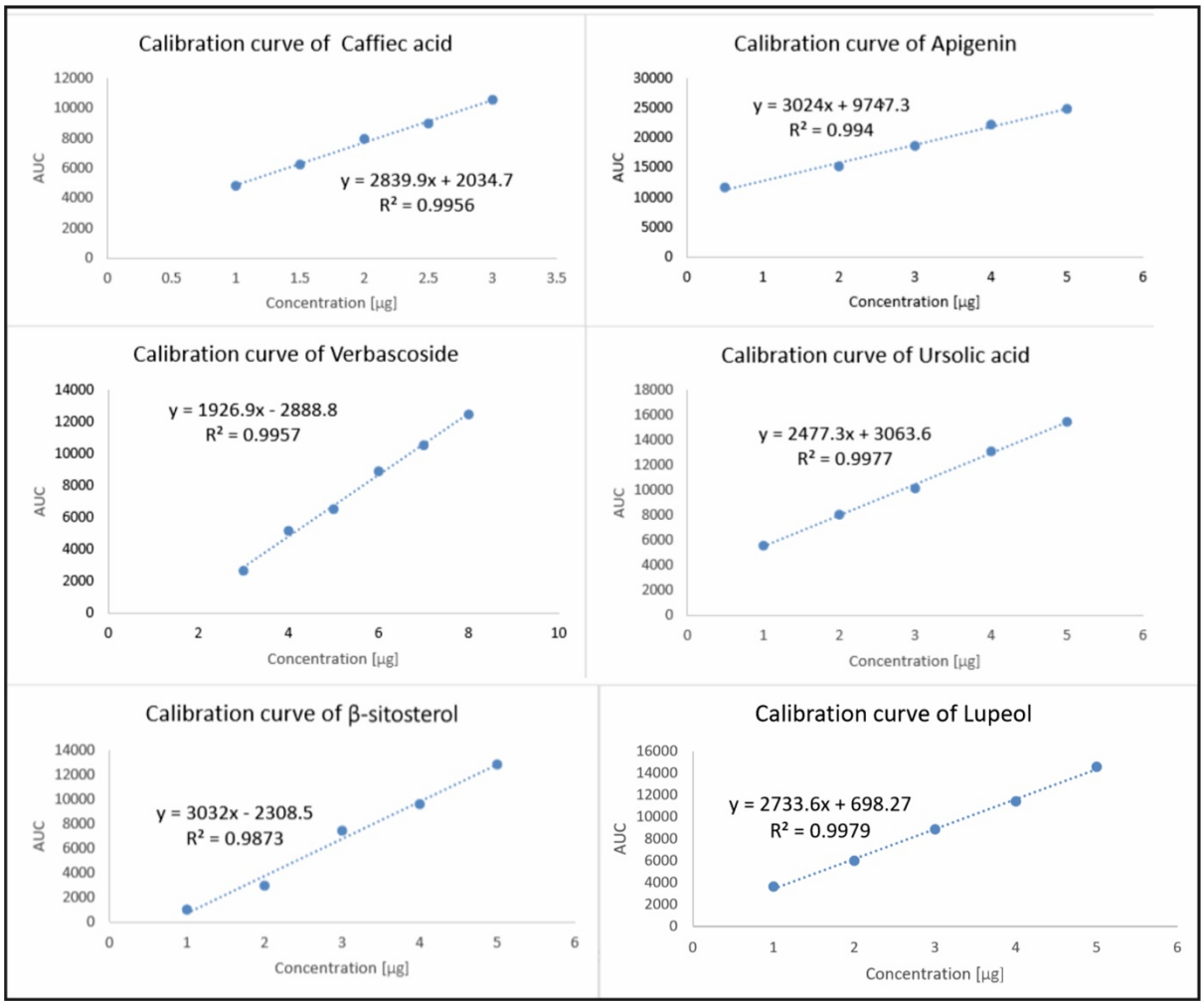

Figure 1: HPTLC densitometric chromatograms.

areas for each phytochemical. The correlation coefficients and regression equations of respective compounds were calculated.

Precision and Repeatability

Precision of the methods were verified from repeatability of measurement and sample application, along with the inter-day and intra-day precision studies. Intra-day and inter-day precisions were examined by analyzing three concentrations of the solutions of standard compounds thrice on the same day as well as on 3 different days, respectively. Repeatability of band applicator were checked by applying bands of the same concentration 7 times and analyzing the 7 tracks for the areas under the curve. The repeatability of the scanner was checked by scanning the same track 7 times for getting areas under the curves. The variability of the peak areas of the test compounds was expressed in terms of % Relative Standard Deviation (% RSD).

Accuracy

Accuracy of the methods were tested by carrying out recovery studies at different spiked level by standard addition method. Standard solutions were added at three different levels (80, 100 and 120%). At each level, three determinations were performed and results were calculated by the difference between the spiked and un-spiked sample analyzed under the same conditions.

LOD and LOQ

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined for each test compound to ascertain the sensitivity of proposed methods. They were measured at signal-to-noise ratios of 3:1 and 10:1, respectively using following formulae:

$$LOD=3.3\ (SD)/SS$$

Table 2: Regression analysis and statistical data of calibration curves.

Compound /Parameter	β-Sitosterol	Lupeol	Ursolic acid	Apigenin	Caffeic acid	Verbascoside
Linearity range (µg / spot)	1 to 5	1 to 5	1 to 5	0.5 to 5	1 to 3	3 to 8
Regression Equation	y=3032x-2308.5	y=2733.6x+698.27	y=2477.3x+3063.6	y=3024x+9747.3	y=2839.9x+2034.7	y=1926.9x-2888.8
Correlation coefficient (r^2)	0.9873	0.9979	0.9977	0.994	0.9956	0.9957
Number of data points	5	5	5	5	5	6

Table 3: Validation data of the developed HPTLC methods.

Compound / Parameter	β-Sitosterol	Lupeol	Ursolic acid	Apigenin	Caffeic acid	Verbacoside
Intraday Precision (% RSD)	0.19-0.71	1.58-1.89	0.29-0.50	0.23-0.49	0.45-0.86	0.45-0.92
Interday Precision (% RSD)	0.21-1.13	2.03-2.86	0.41-1.68	0.24-1.65	0.96-1.73	0.94-1.74
Repeatability of application (% RSD)	0.55	1.02	2.11	0.50	0.88	0.91
Repeatability of measurement (% RSD)	0.48	0.31	0.96	0.38	0.51	0.63
LOD (µg/spot)	0.371	0.410	0.150	0.170	0.348	0.148
LOQ (µg/spot)	0.518	0.855	0.280	0.220	0.855	0.448

Table 4: Results of recovery study for developed methods.

Compound	β-Sitosterol	Lupeol	Ursolic acid	Apigenin	Caffeic acid	Verbascoside
Average% recovery	98.89	98.49	98.39	98.32	98.83	98.97
Range	97.83-99.58	97.77-98.91	98.03-99.04	97.90-98.75	98.20-99.37	98.11-99.74

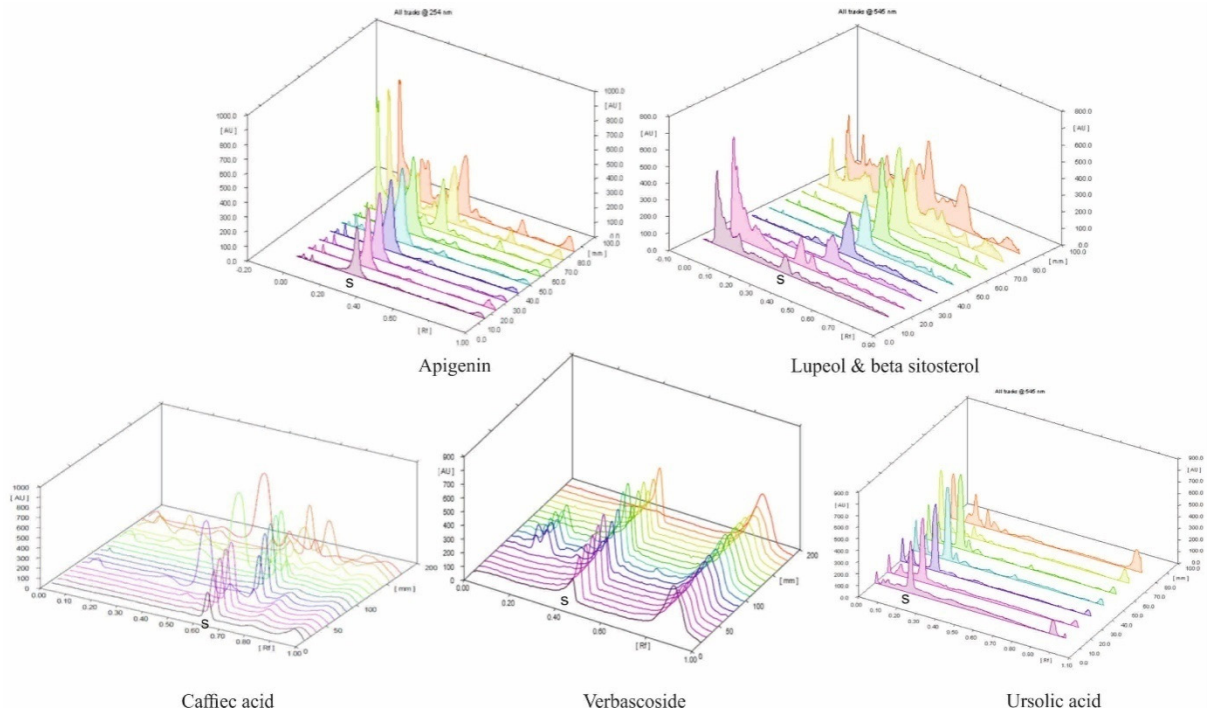

Figure 2: Calibration curves.

$$LOQ=10\ (SD)/SS$$

Where, SD=Standard Deviation of responses and SS=Mean of the slope of the calibration curve.

Specificity

Specificity of the developed methods were determined to ensure that no interferences were obtained from other constituents present in the respective plant extracts. The peak purity of all the analyzed compounds were tested by comparing the UV overlaid spectra and by comparing the spectra at three different levels, viz., peak Start (S), peak apex (M) and peak End (E) positions of the bands.

RESULTS AND DISCUSSION

The phytochemical evaluation is a vital component of quality analysis of a medicinally valued plant. HPTLC is a highly useful analytical method for the identification of medicinal plants through their secondary metabolites as phytochemical markers. In the present study, HPTLC methods were developed for quantification of *β*-sitosterol, lupeol and ursolic acid (in Extract-A), verbascoside (in Extract-B) and apigenin and caffeic acid (in Extract-C). The HPTLC chromatograms and the results of the HPTLC analyses indicating the amounts of important phytochemicals, *β*-sitosterol, lupeol, ursolic acid, apigenin, caffeic acid and verbascoside, in *S. indica* are presented in Figure 1 and Table 1, respectively.

Optimizing the mobile phases that would give the clear separation as well as resolution of bands is one of the challenging tasks to be faced during analysis of plant extracts. Thus, for the chromatographic conditions, several trials were made using different solvent systems. The solvent systems, standardized for the separation of each constituent allowed the determination of these constituents without interference from the other constituents present in the extracts and without causing tailing of the separated bands.

The methods were validated to ensure its credibility through the linearity, accuracy, precision, LOD and LOQ. The linear calibration curves and data for the phytochemicals with coefficients of determination (r2) and respective regression equations are presented in Table 2 and Figure 2.

The results of inter-day and intra-day precisions showed satisfactory accuracy of the methods, with RSD less than 5% in the methods (Table 3). The purity of respective peaks indicated no interference from other constituents present in the respective extracts. The UV overlay spectra ascertained that the proposed methods were specific. The average recoveries (at three different levels) of *β*-sitosterol, lupeol, ursolic acid, apigenin, caffeic acid and verbascoside ranged from 98.83 to 99.72%, the individual results are shown in Table 4. The values indicated that the methods were accurate for the determination of these compounds in *S. indica*. These HPTLC methods therefore are remarkably efficient for the analysis of all the six phytochemicals in *S. indica* plant.

CONCLUSION

The validated HPTLC methods developed here were found to be accurate and precise for the quantitative analysis of *β*-sitosterol, lupeol, ursolic acid, apigenin, caffeic acid and verbascoside in

various extracts of *S. indica*. Extract preparation, mobile phase development and scanning individual marker was taken care to make the methods suitable for quality control of raw materials of *S. indica* plant. The methods developed in the study gave baseline separation indicating the specificity for the tested compounds in the presence of other constituents present in the extracts. In addition, the method for quantification of β-sitosterol, lupeol and ursolic acid allows the simultaneous determination of these two compounds, thus reducing the time, mobile phase solvents and other HPTLC overages.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Stachytarpheta indica is highly valued plant in the Indian traditional system of medicine for treatment of respiratory and digestive complaints. High-performance thin-layer chromatography method is a valuable tool for establishing quality control parameters of raw plant material. In the view of same, present study deals with the development of HPTLC methods for the quantification of phytoconstituents, β-sitosterol, lupeol, ursolic acid, apigenin, caffeic acid and verbascoside, in the whole plant of *S. indica*. The proposed HPTLC methods for estimation of above-mentioned phytochemicals were precise, accurate and selective.

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