Chromatographic Estimation of *p*-Coumaric Acid and Triacontanol in an Ayurvedic Root Drug Patala (*Stereospermum suaveolens* Roxb.)

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Abstract

A high-performance thin layer chromatographic method has been established for the identification and standardization of *Stereospermum suaveolens* extracts using two active biomarkers viz. triacontanol and *p*-coumaric acid in root samples collected from different geographical locations of India. These marker components have different therapeutic activities: *p*-coumaric acid is a good antioxidant and also has anti-diabetic properties; triacontanol has anti-inflammatory and anti-ulcer properties. Studies on root as well as stem samples showed characteristic bands of triacontanol and *p*-coumaric acid at R_f 0.51 and 0.42, respectively. The amount of triacontanol varied from 0.035–8.472 mg/g in the root samples while the amount of p-coumaric acid was in the range 0.081–0.203 mg/g. The limit of detection for triacontanol was 100 ng/spot whereas that for *p*-coumaric acid was 10 ng/spot.

Introduction

Revival of interest in herbal medicines and plant-based medicines is increasing not only in the developing countries but also throughout the world. Therefore, in the present scenario the quality and safety of plant drug has great significance. In this context, a number of parameters have been evolved in which thin layer chromatography (TLC) and high-performance TLC (HPTLC) methods are more reliable and convenient because these methods may be used not only in identification of crude drugs but can also be used for assay and testing of purity, stability, and content of the formulation or batch to batch consistency of herbal products. TLC, along with densitometric determination, can play a significant role for validation of herbal products. Through this, the accuracy, precision, specificity, detection limits, quantification, and linearity can very well be established for quality evaluation and standardization of plant based medicine. Keeping all these aspects in mind, the present work has been planned.

Stereospermum suaveolens (D.C.) is a large deciduous tree, 9–18 m high, with many spreading branches and thick yellowish bark. It is found throughout India in drier localities and is often cultivated (1). In Ayurveda, it is commonly referred to as patala with the root drug having the following properties and actions: rasa (taste): kasaya (astringent), tikta (bitter); guna (physical properties): laghu (lightness), ruksa (dryness); virya (potency): anusna (mildly hot); vipaka (after metabolism): katu (pungent); karma (effect): tridosahara [which subsides all three dosa(s)], rucya (pain).

The therapeutic uses are kapha, which is interpreted as the lypholytic system controlling irreversible degenerative changes in organisms, and vatta, which denotes motor, sensory as well as high central nervous system functions. These therapeutic uses can help with soasa (asthma), sotha (inflammation), asra (piles), chardi (emesis), hikka (hiccup), trsa (thirst), amalapitta (hyperacidity), raktavikara (disorder of blood), mutravikara (urinary disorder), agnidagha, vranariya (ulcer), visphota (blister), and medroga (penile diseases). Hence, the plant finds use in a number of formulations viz. amrtarista, bharangi guda, indukanta ghrta, dhanwantri taila, etc. One of the most important Ayurvedic formulations in which the root is used is Dashmoolarishta (2). It is regarded as a coolant, diuretic, and tonic, generally used in combination with other medicines. The root is bitter, used in inflammations, eructation, vomiting, asthma, fevers, diseases of the blood, thirst, and loss of taste (1).

Although phytochemical studies have been carried out on the stem, leaves, (3) and flowers of *Stereospermum suaveolens*, hardly any chemical constituents have been isolated from the roots (4) .The leaves are reported to contain a number of flavonoids, scutellarein, glucosides such as 6- hydroxyl luteolin-7-galactoside, pigments, and *p*-coumaric acid (3). Lapachol has been isolated from this species, which is reported to have anti-cancer properties when tested on mice (5). Our efforts have led to the characterization and isolation of a saturated alcohol (i.e., triacontanol along with a previously isolated compound *p*-coumaric acid from the roots). Triacontanol has been reported to have anti-inflammatory properties, is effective against carrageenin-induced oedema (6), and also has anti-ulcer effects (7). It is used in combination with octacosanol, dotriacontanol, hexacosanol, and tetracosanol. *p*-Coumaric acid is reported to have anti-oxidant properties (8–13) as well as anti-diabetic action (9–11).

Herbal drug market is becoming more and more competitive day by day due to the ever-increasing demand of the people, and mainly because of the adverse effects of allopathic medicines. Herbal drugs are the preferred drugs due to comparatively low cost, negligible side effects, and easy availability. To further the cause of these drugs, their purity and genuine character needs to

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be confirmed. In order to achieve this aim, certain pharmacognostic and phytochemical parameters need to be established. For most of the plants, their anatomical features have been well-documented, but chemically characteristic components are yet to be identified. For this purpose HPTLC is emerging as the technique with accurate, reproducible, and precise results. A number of workers have reported the separation of many chemical constituents in herbal drugs using this technique (14–17). *p*-Coumaric acid (18) and triacontanol (19) have also been separated in certain plant extracts using HPTLC. Our efforts have led to the identification and determination of two marker constituents, namely triacontanol and *p*-coumaric acid, in the roots of *Stereospermum suaveolens* using HPTLC.

Experimental

Collection and extraction of plant material

Roots of *Stereospermum suaveolens* were collected by Santosh Kumar from different wild locations in India viz. forests of Uttranchal (North Zone), Madhya Pradesh (Central Zone), and Tamil Nadu (South Zone). Their identity was confirmed by matching with the samples in the Herbarium of the institute. The herbarium were deposited in the institute vide voucher specimen number LWG91284A, LWG91284B, and LWG91284C. For the preparation of the extract, the roots were air-dried, powdered, and sieved through 40 mesh. 100 g of the powdered sample was exhaustively extracted thrice with methanol in cold. The extracts were pooled and dried in a vacuum, and amounts of 600 mg, 1700 mg, and 6300 mg were obtained from north, central, and south zone samples, respectively. 10 mg/mL solutions of these extracts were prepared in methanol. The solvent system is composed of the following: toluene–ethyl acetate–formic acid for triacontanol (9:1:0.2, v/v/v); and toluene–ether–acetic acid for *p*-coumaric acid (5:5:0.1, v/v/v). All chemicals and solvents were purchased from M/S Multitrade Instruments and Chemicals Corporation (Lucknow, India).

The system was equipped with a detecting agent anisaldehyde sulphuric acid reagent, HPTLC plates pre-coated silica gel G F254 glass plates were used (20 × 10 cm, 0.2 mm layer thick, Merck, Whitehouse Station, NJ) (Cat. No. 1.05642.0001). The plates were purchased from M/S Gyan Scientific Traders (Lucknow, India). Additional equipment included a Hamilton syringe (100 μL), twin trough glass chamber (Camag, Muttenz, Switzerland), Linomat V (Camag), TLC 3 Scanner connected to Win Cats 3.2 Software (Camag). Camag Reptostar 3 was used to documents the results.

Preparation of calibration curves

10 mg of standard triacontanol (purity ≥ 97%, Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India) was dissolved in 10 mL of chloroform. From this stock solution, 1 mL was diluted with chloroform and volume made up to 10 mL in the volumetric flask. From this solution aliquots of standard triacontanol (100–500 ng) were applied on silica gel G F_{254} plates. 10 mg of standard *p*-coumaric acid (purity ≥ 97%, Sigma Aldrich Chemicals Pvt. Ltd.) was dissolved in 10 mL of methanol. From this stock solution, 1 mL was diluted with chloroform and volume made up to 10 mL in the volumetric flask. From this solution

aliquots of standard *p*-coumaric acid (10–50 μg) were applied on silica gel G F$_{254}$ plates. The plates were developed to a distance of 80 mm in the selected solvent system at room temperature ~ 25°C and at 4°C for triacontanol and *p*-coumaric acid, respectively, because the selected solvent system contains diethyl ether, which is a highly volatile solvent. The plates were dried in a current of warm air and documented in UV 254 nm and 366 nm. Calibration curves were plotted. The plates were scanned in UV at 320 nm for *p*-coumaric acid and at 584 nm for triacontanol before recording the spectra of the two marker components (Figure 1, see page 5A). The plates were dipped in anisaldehyde sulphuric acid, dried, and heated at 110°C for 5 min before scanning and documenting in visible light.

Quantification of the marker component in the samples

20 μL of the sample solutions were applied onto the silica gel plates and the plates processed as outlined previously (Figure 2, see page 5A). The plates were scanned at 584 nm and 320 nm for triacontanol and *p*-coumaric acid, respectively (Figure 3, see page 5A). In order to check the identity of the bands corresponding to the marker constituent, the spectrum of the standards were superimposed on the corresponding band in the sample track (Figure 3). The amounts of triacontanol and *p*-coumaric acid in the samples were calculated from the calibration curves.

Validation of the method

The concept of validation applied to densitometric determinations on HPTLC varies according to the aim of the analysis notably for purity testing (20) and herbals fingerprinting (21). These assay validations are dependent on the spiking of analytes to reconstituted blank matrix (22). International Conference on Harmonization (ICH, 1996) guidelines (CPMP/ICH/381/95; CPMP/ICH/281/95) was followed for the validation of the analytical procedure (23). The method has been validated for precision, accuracy, and repeatability. The same spot was scanned five times to check the precision of the instrument, and the coefficient of variance (CV%) was calculated. The repeatability of the method was established by applying the standards five times and thereafter scanning them so as to calculate the coefficient of variance. In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times following the ICH guidelines. LOD was determined at a signal-to-noise ratio

Table I. Amount of Triacontanol and *p*-Coumaric Acid in the Samples of *Stereospermum suaveolens***

Name of Sample	Triacontanol mg/g	*p*-Coumaric acid mg/g
Root North Zone	0.04 ± 1.61	0.08 ± 2.22
Root Central Zone	1.29 ± 1.55	0.20 ± 1.87
Root South Zone	8.47 ± 1.57	0. 10 ± 2.08
Stem Bark Central Zone	0.04 ± 1.27	0. 75 ± 1.55
Stem Central Zone	1.59 ± 1.44	0.46 ± 1.76
Stem South Zone	1.82 ± 1.38	0.09 ± 2.01
Commercial Sample	0.15 ± 1.63	0.47 ± 1.33

*Values are mean of five determinations with ± SD%.

(S/N) of 3:1 and LOQ at an S/N of 10:1. The peak purity of the markers was assessed by comparing the spectra at three different levels (i.e., peak start, peak apex, and peak end positions of the bands). Recovery studies were carried out by spiking the pre-analyzed samples with extra 50, 100, and 150% of the standard triacontanol and *p*-coumaric acid, and the mixtures were re-analyzed by the proposed method. The experiment was conducted six times and CV% was calculated.

Results and Discussion

Keeping in view the aspect of drug authentication and standardization for the preparation of effective herbal formulations, an HPTLC method has been developed for the identification and quantification of chemical marker components in the roots of *Stereospermum suaveolens*. Triacontanol and *p*-coumaric acid have been determined in different root samples studied. Of all the solvent systems tried, two solvent combinations were finalized for the quantitative determination of these markers: toluene–ethyl acetate–formic acid (9:1:0.2, v/v/v) and toluene–ether–acetic acid (5:5:0.1, v/v/v) for triacontanol and *p*-coumaric acid, respectively.

The plates were scanned at 584 nm and 320 nm for triacontanol and *p*-coumaric acid, respectively (Figure 3) from where the presence of the two marker components can be evaluated. The LOD and LOQ of these two markers are given in Table IV. The R_f values of triacontanol and *p*-coumaric acid were 0.51 (Table II) and 0.42 (Table III), respectively. The amount of triacontanol ranged from 0.035–8.472 mg/g in the root samples studied whereas *p*-coumaric acid was in the range 0.081–0.203 mg/g (Table I).

The regression lines of standards pass through origin resulting in minimal systematic errors and minimizing the difference in the signal of the analysis and the standard (22,24). The use of multiple level calibrations each time for analysis is thought to be the governing factor for these observations (25). The linear regression equations for triacontanol and *p*-coumaric acid were $y = 46.854x + 0.029$, $r = 0.99725$ and $y = 623.271x + 146.274$, $r = 0.99770$, respectively, with standard

deviation being 0.54 and 3.67%. The recovery of the marker components has been calculated via spiking experiments. The results are given in Tables V and VI.

The method has been extended to evaluation of stem samples as well as commercial samples. The amount of triacontanol in stem samples ranged from 0.042–1.819 mg/g while *p*-coumaric acid was found to be present in the range 0.087–0.456 mg/g. The amount of the two markers was 0.03 and 0.09 mg/g, respectively, in the commercial sample analyzed.

Figure 2 represent the general profile of all the samples along with triacontanol and *p*-coumaric acid as marker components. The R_f values of all the spots visible on the plate are tabulated in Tables II and III. As is apparent from the Figure 2A, some common bands are visible in the tracks of the root as well as stem samples. A dark green spot at R_f 0.10, a purple one at 0.16, a green at 0.23 and 0.30, purple at 0.68 and 0.76 are the common bands found in all the samples. A distinguishing prominent blue band is seen at R_f 0.57 in the samples from the southern region of the country. The same compound seems to

Table III. R_f values of the Chemical Constituents Visible After Derivatization with Anisaldehyde Sulphuric Acid Reagent

R_f, Color	T 1*	T 2[†]	T 3[‡]	T 4[§]	T 5**	T 6[††]	T 7[‡‡]	T 8[§§]
0.09 (Purple)	+	+	+	+	+	+	+	–
0.14 (Yellow)	–	+	+	+	–	–	–	–
0.19 (Purple)	+	+	–	+	+	+	+	–
0.27 (Yellow)	–	+	+	+	–	–	–	–
0.33 (Green)	+	+	+	+	+	–	+	–
0.42 (pink) (p-coumaric acid)	+	+	+	+	+	+	+	+
0.47 (Purple)	+	+	+	+	+	+	+	–
0.51 (Blue)	+	–	–	–	–	–	+	–
0.54 (Blue)	–	+	–	+	–	–	–	–
0.59 (Purple)	–	+	+	–	+	–	+	–
0.66 (Yellow)	+	+	–	+	+	+	+	–
0.72 (Purple)	+	+	+	+	–	+	–	+
0.83 (Purple)	+	–	–	–	+	+	+	+

* Track 1: Root Northern Region.   [†] Track 2: Root Central Region.
[‡] Track 3: Root Southern Region.   [§] Track 4: Stem Southern Region.
** Track 5: Stem Central Region.   [††] Track 6: Stem Northern Region.
[‡‡] Track 7: Market Sample.   [§§] Track 8: Marker Component.

Table II. R_f Values of the Chemical Constituents Visible After Detection with Anisaldehyde Sulphuric Acid Reagent

R_f, Color	T 1*	T 2[†]	T 3[‡]	T 4[§]	T 5**	T 6[††]	T 7[‡‡]	T 8[§§]
0.10 (Dark green)	+	+	+	+	+	+	+	–
0.16 (Purple)	+	+	+	+	+	+	+	–
0.23 (Green)	+	+	+	+	+	+	+	–
0.30 (Green)	+	+	+	+	+	+	+	–
0.39 (Blue)	–	+	+	+	–	–	–	–
0.47 (Purple)	+	+	+	+	+	+	+	–
0.51 (Purple) triacontanol	+	+	+	+	+	+	+	+
0.57 (Blue)	–	+	+	+	–	–	–	–
0.63 (Yellow)	+	+	+	+	+	–	+	–
0.68 (Purple)	+	+	+	+	+	+	+	–
0.76 (Purple)	+	+	+	+	+	+	+	–
0.87 (Purple)	+	–	–	–	+	–	–	+
0.92 (Purple)	–	+	+	+	+	+	+	–

* Track 1: Root Northern Region.   [†] Track 2: Root Central Region.
[‡] Track 3: Root Southern Region.   [§] Track 4: Stem Southern Region.
** Track 5: Stem Central Region.   [††] Track 6: Stem Northern Region.
[‡‡] Track 7: Market Sample.   [§§] Track 8: Marker Component.

Table IV. Linearity Studied Using Validation Scheme*

Compound	Triacontanol	*p*-Coumaric acid
Standard Solution		
Determination coefficient r^2	0.99725	0.99770
Equation of straight line	$y = 46.854x + 0.029$	$y = 623.271x + 146.274$
Standard deviation	0.54	3.67
CV%	1.92	0.39
n	6	6
LOD (ng)	100	10
LOQ (ng)	693	82
Spiked *Stereospermum suaveolens*		
Determination coefficient r^2	0.99529	0.99880
Equation of straight line	$y = 8002.899x + 51.749$	$y = 766.026x + 10.896$
Standard deviation	3.81	3.90
CV%	1.47	1.58
n	6	6

* Regression on data from three experiments. Measurements corrected by internal standardization, UV scan at 280 nm; visible: post-chromatographic derivatization with sulphuric acid–anisaldehyde reagent and scan at 584 nm.

Table V. Recovery Studies (*n* = 6) for Triacontanol

Triacontanol added (%)	Theoretical content (ng)	Amount found (ng)	Recovery (%)	RSD (%)	SE
0	400	385	96	0.13	1.12
50	600	596	99	0.35	1.16
100	800	788	98	0.37	1.28
150	1000	982	98	0.25	1.76

Table VI. Recovery Studies (*n* = 6) for *p*-Coumaric Acid

Triacontanol samples (%)	Theoretical content (ng)	Amount found (ng)	Recovery (%)	RSD (%)	SE
0	400	393	98	0.20	1.58
50	600	595	99	0.15	1.26
100	800	789	99	0.18	1.22
150	1000	978	98	0.24	1.74

be present in the root sample collected from central region of the country. Another blue band is seen at R_f 0.39 in these three samples. Another characteristic band seen in the samples is a yellow one at R_f 0.63, which is absent in the stem collected from central region of the country.

From the Figure 2B, again a few distinguishing bands can be visualized. Two common yellow bands seen in the root sample from central India and the root and stem samples from the southern region are visible at R_f 0.14 and 0.27. A blue spot is seen again in these three tracks, which is absent in the other tracks at R_f 0.54. In the root sample from the south the yellow band at R_f 0.66 is found to be absent.

From the foregoing discussion, it can be observed that there are a few compounds, though not identified as yet, on the basis of which the samples from the south can be distinguished. The difference in the bands can be attributed to the environmental variations. Though as a rule, there are hardly any separate compounds present in the root or the stem of *Stereospermum suaveolens*. Hence, it can be said that the substitution of the root drug with the stem or the stem bark of the plant may not cause much variation in the medicinal properties of the drug. As yet there is no conclusive evidence for the same.

Conclusion

It can now be concluded that triacontanol, which is anti-inflammatory in nature, is present in the root of *Stereospermum suaveolens* in appreciable quantity, thereby justifying the use of this plant in dashmoolarishta, which imparts this effect as well as other therapeutic benefits. Although its presence is noted in the stem samples also, its amount is comparatively less. The presence of *p*-coumaric acid is noted in all the root as well as stem samples studied. From the results, it can be inferred that the HPTLC method developed is not only suited for raw herbal drugs but can also be extended to the estimation of these compounds in fruits, vegetables, and the formulated products as well.

For the preparation of authentic herbal formulations, the raw material (i.e., the plant material used should be genuine and authenticated). To obtain genuine material, some chemical standards along with botanical identification, need to be established because botanical identification alone is not suitable in case of dried or powered plant material. Hence, seeing the applicability of this method to a wide range of materials, it can be finally concluded that HPTLC has again proved to be an effective technique for herbal drug industry.

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