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Standardization of Marketed Adulsa Syrup Containing Vasaka by High Performance Thin Layer Chromatography

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Adulsa syrup is a marketed Ayurvedic formulation containing vasaka as one of the main ingredient. Present study aims to standardize this formulation by HPTLC finger printing of vasicine fraction, isolated from authenticated powder of *Adhatoda vasica* and characterized by IR, UV and HPLC. Three extracts, namely aqueous extract with 5 % citric acid, acetone:alcoholic (1:1) extract and alcoholic extract 90 % of powder have been prepared and evaluated. Acetone:alcohol (1:1) extract indicated presence of maximum amount of vasicine. Densitometric scan showed three well-resolved peaks at 254 nm. The central peak was integrated as vasicine. The other two peaks were ketone which was proved by dinitrophenylhydrazine test. Standard calibration curve was prepared using isolated vasicine fraction as a marker. Calibration plots were linear in the range 4-12 µg/spot with correlation coefficient 0.9982. Recovery of vasicine was 103.82 %. Stress stability study of vasicine was carried out using acid, alkali and photodegradation. The suitability of this HPTLC method for quantitative determination of compound was proved by validation in accordance with the requirements of pharmaceutical regulatory standards.

Key Words: Standardization, Vasaka powder, Vasicine, HPTLC.

INTRODUCTION

Ayurveda, the ancient Indian system of medicine, strongly believes in polyherbal formulations and scientists of modern era often ask for scientific validation of herbal remedies. This plant-based system of medicine has already gained worldwide attention due to its safety and efficacy. With the growing need for safer drugs, attention has been drawn to quality, efficacy and standards of the Ayurvedic formulations[1,2]. Ayurvedic pharmacy advocates the use of quality control tests to make sure that the prepared medicines adhere to the standards mentioned in Ayurveda. Most of the tests described in ancient literature appear to be based on observation and seem subjective without valid scientific backing. Hence standardization and development of reliable quality protocols for Ayurvedic formulations using modern techniques of analysis is extremely important[3]. Ayurveda is a plant-based system of medicine and consist of various Ayurvedic formulations such as solid dosage forms (pills, powders), liquid dosage forms (asavas, aristhas) and semisolid dosage forms (ghritas, avlehas). Syrups are medicinal preparations made by soaking the drugs (powder or decoction) in a solution of sugar or jaggery for a specified period of time. Adulsa is a marketed Ayurvedic polyherbal formulation included in Ayurvedic formulary containing vasaka as the main ingredient. Vasaka formulations were selected because it is most widely used in the treatment of cough and cold[4-11].

The present investigation is concern with the isolation and characterization of vasicine from authenticated powder of *Adhatoda vasica* and standardization of formulations. Two marketed Adulsa syrup (Vasaka) was taken for standardization. Thus, the present study aims to develop a HPTLC method for standardization of marketed ayurvedic formulations containing vasaka.

EXPERIMENTAL

All the solvents purchased from E. Merck and S.D. Fine Chemicals, Mumbai. All solvents used for extraction, TLC and HPTLC studies were distilled before use. Solvents used for UV and IR studies were of spectroscopic grade. Solvents used for HPLC analysis were of HPLC grade. Precoated silica gel 60 F_{254} plates procured from E. Merck, Mumbai were used for TLC and HPTLC studies. The UV spectra were recorded on a Shimadzu, UV-2450, V 2.21 double Beam UV spectrophotometer. HPTLC studies were carried out using CAMAG Linomat V applicator, a Camag twin trough TLC chamber, a Camag TLC scanner 3, Camag Wincats software V 1.4.2 and

a Hamilton (Reno, Nevada, USA) Syringe (100 µL). The HPLC analyses were done on a JASCO PU-980 system. All the results were obtained by repetition of the each experiment at least three times. Commercially available formulation of Adulsa syrup was procured from local market.

Standardization using physicochemical parameters: The sample of vasaka was analyzed for various parameters such as, determination of alcohol soluble extractive, water soluble extractive, loss on drying, total ash value, acid insoluble ash value, water soluble ash value and determination of heavy metals[12] (Table-1).

TABLE-1
ASH VALUES OF FORMULATION AND RAW MATERIAL

S. No.	Type of ash (%)	Formulation	Raw materials
1	Total ash	4.89	13.49
2	Acid insoluble ash	0.23	2.10
3	Water soluble ash	3.99	8.66

Preparation of extracts: Three types of extracts namely aqueous extract with 5 % citric acid, acetone:alcoholic (1:1) extract and alcoholic extract 90 % were obtained by macerating the vasaka powder with the respective solvents for 15 days and the extracts were dried (cold maceration process).

Isolation of vasicine: Aqueous extractives were dissolved in 2 % sulphuric acid. Partitioned with chloroform. The chloroform fraction was discarded. The aqueous phase was basified with 10 % ammonia solution and extracted with chloroform. The extract was purified for vasicine using alcohol and ether. Further the vasicine obtained was purified by column chromatography using aluminium oxide (active basic, pH 8.5 to 9.5) as adsorbent. Same procedure was used for the other two extracts[5] (Table-2).

TABLE-2
WEIGHT OF THE EXTRACTS AND AMOUNT OF ISOLATED VASICINE

Weight of vasaka powder (g)	Type of cold Maceration	Weight of extract obtained (g)	Amount isolated vasicine and vasicinones (g)
300	Acetone:Alcohol (1:1)	0.8612	0.1250
300	Alcohol 90 %	0.5525	0.1480
300	Aqueous with 5 % citric acid	0.1756	0.3247

All the extracts were qualitatively evaluated by chemical tests and TLC studies for the presence of various phytoconstituents like alkaloids, carbohydrates, phenolic compounds, tannins, phytosterols, glycosides, proteins amino acids and flavonoids[13,14] (Table-3).

UV and IR spectra were recorded for Middle fraction. UV spectra were recorded in methanol and ethanol. An IR spectrum of neat sample was recorded.

Middle fraction indicated presence of vasicine which is reported to be a major component of vasaka. It was further analyzed by HPLC using following conditions: Column: C18 (25 cm × 4.6 mm, ad.) and 5 µ particle size; Mobile phase:methanol: water (70:30); detection at 254 nm; flow rate: 1 mL/min.

TABLE-3
QUALITATIVE CHEMICAL EVALUATION OF EXTRACTS AND FORMULATIONS

Phytoconstituent	Extracts			Formulation
	Alcohol: acetone (1:1)	Alcohol 90 %	Aqueous with 5 % citric acid	
Alkaloids	+	+	+	+
Carbohydrates and glycosides	–	–	–	+
Phytosterols	+	+	+	–
Phenolic compounds and tannins	–	–	–	+
Proteins and amino acids	–	–	–	–
Flavonoids	+	+	+	–

(+) Indicates presence, (–) indicates absence.

TLC studies: TLC studies of all the extracts were carried out using silica gel 60 F_{254} precoated plates as stationary phase and methanol:toluene:dioxane:ammonia (2:2:5:1) as mobile phase. Spots were observed under UV and visible light. Isolated fraction of extracts showed three peaks in HPTLC chromatogram. First and third fraction showed solid separation with dinitrophenylhydrazine test and were proved to be ketone. The middle fraction showed no separation of solid and was proved to be vasicine (R_f 0.74). The structure of purified fraction of vasicine was characterized by UV, HPLC and IR analysis.

HPTLC studies: HPTLC analysis of formulation for quantification of vasicine by using isolated vasicine as a standard. High performance thin layer chromatography fingerprint of all extracts was recorded at 254 nm.

HPTLC method for estimation of vasicine

Preparation of calibration curve of standard vasicine: 25 mg of standard vasicine was dissolved in 25 mL of ethanol to yield stock solution 1000 µg/mL. Calibration curve from 4-12 µg/spot was prepared and checked for linearity. The concentration range for calibration curve was decided on the basis of probable concentration of vasicine in the pharmaceutical herbal formulation.

Sample preparation: Each 10 mL of the Adulsa syrup was extracted with methanol, the mixture was concentrated and the residue (12 mg) was dissolved in methanol (10 mL). 10 µL of each formulation used for quantitative estimation.

Chromatographic conditions: Chromatography was performed on 10 cm × 20 cm aluminium backed silica gel 60 F_{254} TLC plates (Merck, Darmstad, Germany). Before use the plates were washed with methanol then dried in an oven at 50 ºC for 5 min. Samples were applied as 6 mm bands by spraying at a rate of 15 s μL^{-1} by means of a Camag (Muttenz Switzerland) Linomat V sample applicator equipped with a 100 µL syringe (Hamilton, Reno, Nevada, USA); the distance between the bands was 13.0 mm. Ascending development of the plate, migration distance 70 mm, was performed at 25 ± 2 ºC with methanol:toluene:dioxane:ammonia (2:2:5:1) as mobile phase in a Camag twin-trough chamber previously saturated for 20 min. The average development time was 20 min. After development the plate was dried at 50 ºC in an oven for 5 min. The spots were scanned at λ_{max} 254 nm.

Validation of the HPTLC method: HPTLC method development for quantitative determination of vasicine in marketed formulation[15-24].

Linearity: Amount of isolated vasicine fraction applied was equivalent to 4-12 µg per spot. Vasicine were applied to a prewashed TLC plate. The plates was developed, dried and scanned as described above. A calibration plot was constructed by plotting peak area against amount of vasicine fraction (µg per spot). The linearity of response for vasicine fraction was assessed in the concentration range 4-12 µg per spot. The slope, intercept and correlation coefficient were also determined. Over the concentration range studied, the correlation coefficient for the calibration plot was r = 0.9982 and the slope = 359.30 (n = 5). The results are listed in Table-4.

TABLE-4
LINEARITY DATA

Parameters	Vasicine
Beer's law limit (µg/spot)	4-12
Correlation coefficient	0.992
Intercept	219.9
Slope	774.9

Analysis of marketed formulation: The spots at R_f 0.74 for vasicine were observed in the densitogram of the samples extracted from formulation. There was no interference from the excipients commonly present in the formulation. The vasicine content was found to be 102.75 % (Table-5).

TABLE-5
ESTIMATION OF VASICINE IN FORMULATIONS

	Vasicine
Labeled claim (mg)	1.6
Amount found (%) ± SD (n = 5)	102.75 ± 1.89
% RSD	1.84

Sensitivity: The sensitivity of measurement of vasicine by the use of the proposed method was estimated in terms of the limit of quantitation (LOQ) and the lowest concentration detected under the chromatographic conditions as the limit of detection (LOD)[22-26]. The LOQ and LOD were calculated by the use of the equations LOD = 3 × N/B and LOQ = 10 × N/B where N is the standard deviation of the peak areas of the drug (n = 3), taken as a measure of noise and B is the slope of the corresponding calibration plot. The limit of quantitation (LOQ) was 15 µg and the limit of detection (LOD) was 4 µg.

Precision: The precision of the method was carried out by spotting the samples of 4 µg of standard vasicine fraction four times on pre-coated TLC plate by using Linomat V applicator with methanol:toluene:dioxane:ammonia (2:2:5:1) as mobile phase.

Accuracy: Recovery studies were carried out by using 4 µg of the methanol extracted formulation and the TLC plate was spiked with 4 and 6 µg of standard vasicine isolated from the acetone:alcohol (1:1) extract. The plate was developed in a similar manner and amount of vasicine was quantified as described above. The data was analyzed for the amount of vasicine recovered. This estimation was repeated three times.

Specificity: The mobile phase designed for the method resolved the drug very efficiently, as shown in the Fig. 1. The R_f value of vasicine 0.74. A typical absorption spectrum of vasicine is shown in Fig. 2. The wavelength 254 nm was selected for detection because it resulted in better detection sensitivity for the drug. The spot for vasicine from the syrup formulation was identified by comparing its R_f value and its absorbance/reflectance spectrum with those of standard vasicine. The peak purity of vasicine was tested by comparison of spectra acquired at the peak-start (S), peak-apex (A) and peak- end (E) positions of the spot. The correlation between these spectra were indicative of the purity of vasicine peak (correlation r (S, M) = 0.999, r (M, E) = 0.998), as shown in Fig. 3.

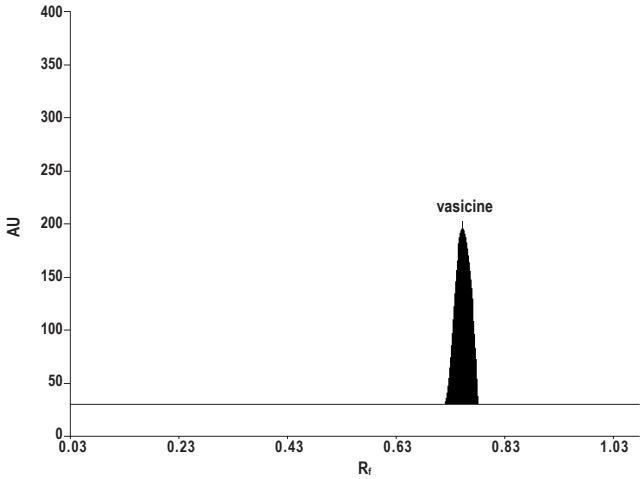

Fig. 1. Typical HPTLC Chromatogram of vasicine, measured at 254 nm, mobile phase methanol:toluene:dioxane:ammonia (2:2:5:1 v/v)

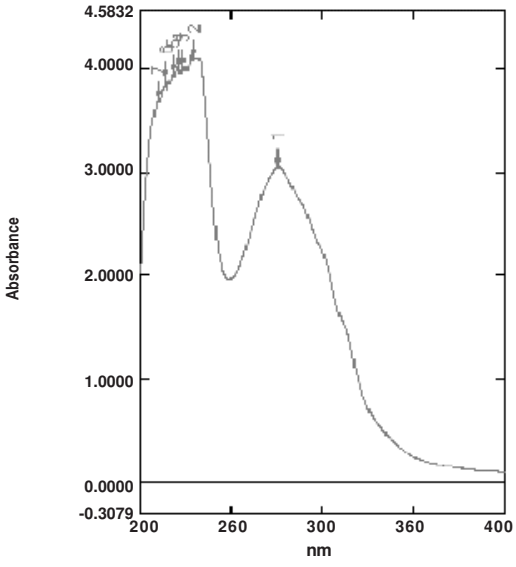

Fig. 2. UV-Visible spectra of vasicine fraction

Batch to batch variation: Formulation of two batches (batch no. 147 and 247) 10 µL each was spotted on pre-coated TLC plate by using Linomat V applicator with methanol: toluene:dioxane:ammonia (2:2:5:1) as mobile phase. Calibration curve in the range 4-12 µg/µL was prepared and the area was recorded.

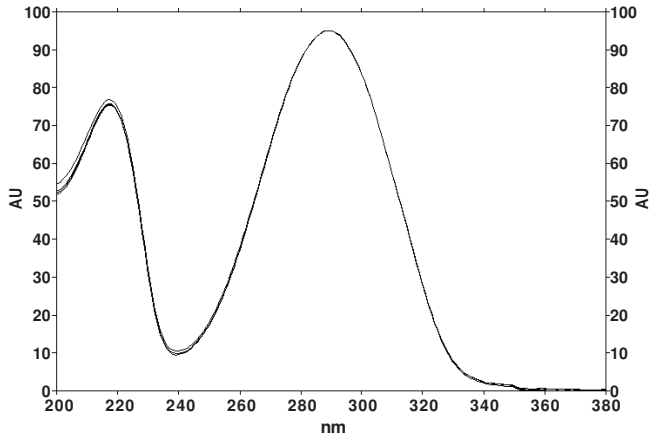

Fig. 3. Peak purity spectras of vasicine extracted from a powder of *Adhatoda vasica*, scanned at the peak-start, peak-apex and peak-end positions of the spot (correlation > 0.99)

Stress-stability studies of standard vasicine: A stock solution prepared as 1000 µg/µL of vasicine in methanol was used for forced degradation studies.

[I] Acid hydrolysis: 25 mL of 1 % standard solution of vasicine was taken in the round bottom flask. To it, 10 mL of 0.1 N HCl solution was added and reflux for 8 h on water bath.

[II] Alkali hydrolysis: 25 mL of 1 % standard solution of vasicine was taken in the round bottom flask. To it, 10 mL of 0.1 N NaOH solution was added and reflux for 8 h on water bath.

[III] Oxidation: 25 mL of 1 % standard solution of vasicine was treated with 1 % hydrogen peroxide solution and reflux for 8 h on water bath.

[IV] Photolytic degradation: 25 mL of 1 % standard solution of vasicine was exposed to bright sunlight for 24 h.

RESULTS AND DISCUSSION

Standardization of Adulsa syrup containing vasaka as per pharmacopoeia was carried out based on the physicochemical parameters[7]. A summary of the qualitative analysis is given in Table-2. In the present study, raw materials were subjected to various pharmacognostic evaluations. The raw material was authenticated by the powder characteristics of the drug which showed the presence of diacytic stomata, covering and glandular trichomes, xylem vessels with annular to spiral thickening and lignified, cystoliths and calcium oxalate crystals in acicular and prismatic form. The total ash content was found to be 13.49 % w/w and acid insoluble ash was 2.10 % w/w, which is within the prescribed limit. The result of total ash value indicated that there is no contamination, substitution or adulteration in the crude drug. Moisture content of the drug obtained was 5.01 % w/w which indicated quality of crude drug. The various extractive values of vasicine and vasicinone isolated from acetone:alcohol (1:1) was 3.24 g, 90 % alcohol was 1.48 g and from aqueous was 1.25 in 300 g of powders, respectively. Heavy metals like iron, lead, arsenic were absent in formulation. For this study vasicine was isolated from authenticated powder of *Adhatoda vasica* and characterized by UV and HPLC with retention time 3.433 min (Figs. 2 and 4). IR peaks observed at 3792, 2997, 2180, 1548, 1218, 810 cm^{-1} for OH-strech, C-H stretch, conjugated cyclic stretch, C-O stretch and C-N stretch, respectively. For TLC densitometric quantification of vasicine, preliminary TLC fingerprinting was carried out in order to optimize the mobile phase to obtain a clearcut separation of the band of vasicine from the rest of the compounds. The mobile phase was optimized, methanol: toluene:dioxane:ammonia (2:2:5:1) resolved vasicine at R_f 0.74. Identification of the band of vasicine in the sample extract was confirmed by overlaying the UV spectra with that of respective standard using CAMAG TLC scanner III. The developed TLC densitometric method for the estimation of vasicine was validated in terms of precision and accuracy. The linearity range for vasicine was found to be 4 to 20 µg/spot with correlation coefficient r, 0.9982. The results of the linearity studies are shown in Table-4. The % RSD values for precision are depicted in Table-6. The limit of detection for vasicine was found to be 0.8 µg and the limit of quantification was found to be 20 µg.

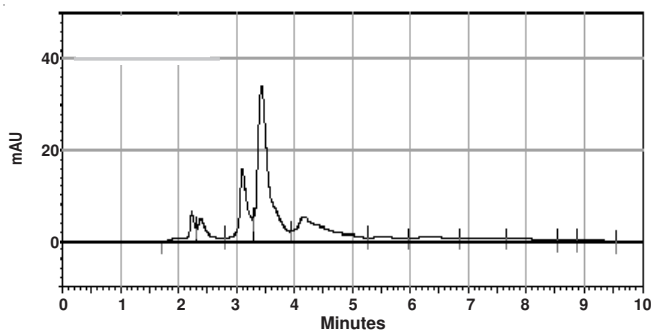

Fig. 4. HPLC of vasicine developed in methanol:water (7:3)

TABLE-6
PRECISION OF VASICINE

S. No.	Application volume (µL)	Area of vasicine (4 µg)
1	4	1701.41
2	4	1700.34
3	4	1770.07
4	4	1712.90
5	4	1743.68
	Mean	1751.74
	SD	31.37
	% RSD	1.81

The per cent recovery at three different levels was found to be 99.5-106.94 and the results are listed in Table-7. HPTLC data revealed that two batches of formulation, batch number 147 and 247, showed batch-to-batch variation in their vasicine content (4.962 µg and 1.497 µg, respectively). In Batch no 247 amount obtained was below LOD. The results are listed in Table-8. To check stability of isolated vasicine fraction, HPTLC stability studies performed and was degraded in acid, alkali and hydrogen peroxide. As well as photolytic degradation of the vasicine after exposure to 24 h of bright sunlight.

TABLE-7
RESULTS OF RECOVERY STUDY

	Addition of vasicine (%)	Initial amount (ng)	Recovery (%)	% RSD
Vasicine	50	4000	99.55	0.48
	100	4000	99.26	0.02
	150	4000	106.94	1.21

TABLE-8
BATCH TO BATCH VARIATION

Formulation brand 1 Batch No.	Volume applied (µL)	Area	Amount obtained (µg)
147	10	1611.16	4.962
247	10	811.68	1.497

Thus the vasicine was decomposed under forced stress conditions and also on exposure to light. The results are listed in Table-9.

TABLE-9
DEGRADATION TRIAL FOR VASICINE

Condition	Time (h)	R_f	Area	Recovery (%)
Acid, 0.1 N HCl, 80 ºC	8	0.73	6410.89	52.60
Base, 0.1 N NaOH, 80 ºC	8	0.73	5780.36	47.42
H_2O_2, 1 % at room temperature	8	0.74	10789.39	88.52
Daylight heat 30 ºC	24	0.74	7923.24	65.01

Conclusion

The spectral data and HPTLC fingerprint of all extracts of vasaka powder which is separated for vasicine and vasicinone could be used as a marker for standardization of Adulsa syrup. HPTLC fingerprinting is the proper analytical tool in the routine standardization of Adulsa syrup to check the batch to batch variation. Vasicine can be used as one of the appropriate analytical markers for standardization of Adulsa syrup.

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