Indian Journal of Natural Products and Resources
Vol. 7 (4), December 2016, pp. 301-309

Evaluation of *Shaddharana churna*: An *Ayurvedic* formulation

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Received 26 January 2016; Revised 12 July 2016

Shaddharana churna is a well-known *Ayurvedic* formulation that finds its reference in renowned *Ayurvedic* treaties such as *Ashtangasangraham* and *Ashtanghridaya*. As the name suggests *Shaddharana churna* is composed of equal parts of six drugs namely *Katuka*, *Daruharidra*, *Ativisha*, *Chitraka*, *Patha*, and *Indravaya*. Evaluation of *Ayurvedic* formulations is necessary to ensure their quality, strength, purity, and authenticity. Present work deals with microscopic, physico-chemical, qualitative, and quantitative evaluation of *Shaddharana churna*. Qualitative and quantitative evaluation was done by development and validation of high performance thin layer chromatography using berberine from *Daruharidra*, apocynin from *Katuka* and plumbagin from *Chitraka* as marker compounds. The outcomes of the research conform to the need of ensuring quality and safety of *Ayurvedic* medicines.

Keywords: Apocynin, Berberine, HPTLC, Plumbagin, *Shaddharana churna*.

IPC code; Int. cl. (2015.01)−A61K 36/00

Introduction

Ayurvedic formulations are prepared in a number of dosage forms, most of which are polyherbal formulations (PHFs) i.e. formulations prepared by using more than one drug. PHFs theoretically produce greater result when compared to an individual use of the plant and also the sum of their individual effect[1]. Evaluation and standardization of such polyherbal *Ayurvedic* medicines is a highly researched topic in drug industry today. Lack of quality standards in the preparation of *Ayurvedic* medicines may result in mild to severe adverse effects. Therefore, standardization of PHFs is essential in order to assess the quality of drugs based on the concentration of their active phytochemicals i.e. markers[2].

Present work deals with spectroscopic as well as pharmacognostic evaluation of *Shaddharana Churna*, an *Ayurvedic* PHF. *Churna* is a type of dosage form in *Ayurvedic* system of medicine. It is a mixture of fine powder of drug(s). Drugs are cleaned, dried, pulverised, and sieved to obtain a uniform *churna*. These forms of medicament are prescribed generally because of their particle size. Smaller the particle size greater is the absorption rate from gastrointestinal tract and hence, greater is bioavailability[3]. *Shaddharana churna* finds its reference in *Ayurvedic* treaties such as *Ashtangasangraham* and *Ashtanghridaya*. The formulation is prescribed mainly against *vata dosha*, bloating and a complex condition *Amashayagata vata*[4], neuromuscular diseases such as ankolysing spondylitis[5], Meniere's disease[6], leprosy, hemorrhoids, diabetes, and anaemia[7].

As the name suggests *Shaddharana churna* consists of equal quantity of powders of 6 medicinal plants namely, *Chitraka* (*Plumbago zeylanica* L.), *Indravaya* (*Holarrhena antidysenterica* (L.) Wall), *Patha* (*Cyclea peltata* Hook. f. & Thomson), *Katuka* (*Picrorrhiza kurroa* Royle ex Benth.), *Ativisha* (*Aconitum heterophyllum* Wall. ex Royle), and *Daruharidra* (*Berberis aristata* DC)[4].

Shaddharana churna is a well-known and regularly prescribed *Ayurvedic* formulation and therefore, needs to be standardized with respect to its quality, purity, identity, and strength. Comparative studies between 3 formulations were performed, two being marketed formulations (named as M1 and M2 for the study) and one in-house formulation. The formulation under study was standardized by simultaneous estimation of berberine, apocynin, and plumbagin as marker compounds using HPTLC. Berberine, apocynin, and plumbagin are present in *Daruharidra*, *Katuka* and *Chitraka* respectively[8-10].

Estimation of berberine and plumbagin individually from their respective botanical sources

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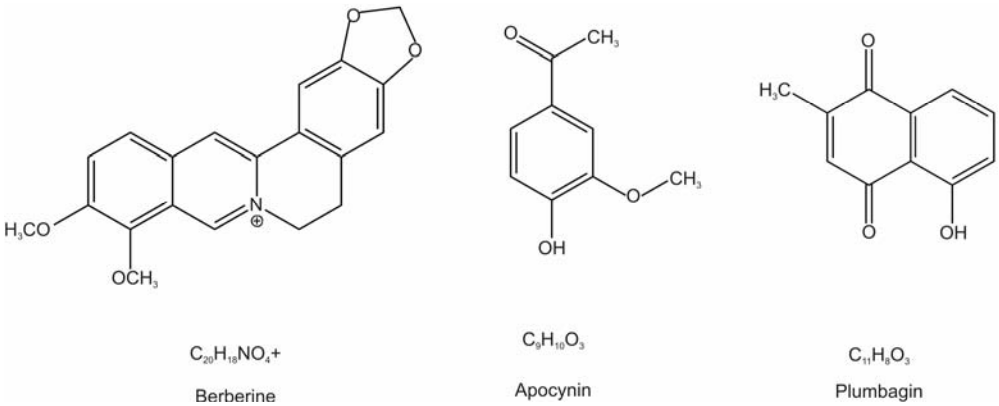

Fig. 1-Structure of the marker compounds

has been reported in the literature[11,12]. However, simultaneous estimation of berberine, apocynin, and plumbagin has not been studied. Structure of the marker compounds are given in Fig. 1.

Materials and Methods

Raw materials and marketed formulations

Marketed formulations of *Shaddharana churna* from two well-known brands M1 and M2 were procured from local market of Mumbai, Maharashtra, India. An in-house formulation was prepared using drug powders procured from Yucca Enterprises, Mumbai, Maharashtra, India. All the raw materials used for preparation of in-house formulation were authenticated from taxonomist Dr H M Pandit (Ex-Head and Associate Professor of Botany, Department of Botany, Guru Nanak Khalsa College, Mumbai). Voucher specimens namely ba 840616 (*Berberis aristata* DC), ah 840516 (*Aconitum heterophyllum* Wall. ex Royle), cp 840316 (*Cyclea peltata* Hook. f. & Thomson), ha 840216 (*Holarrhena antidysenterica* (L.) Wall), pk 840416 (*Picrorhiza kurroa* Royle ex Benth.), and pz 840116 (*Plumbago zeylanica* L.) were deposited in Department of Quality Assurance at Bharati Vidyapeeth's college of Pharmacy, Navi Mumbai. The particle size of the formulation was 80 mesh.

Standards and reagents

All the chemicals of AR grade were procured from S D Fine chemicals, Mumbai. Analytical standards, apocynin, and berberine were purchased from Yucca Enterprises, Mumbai. Standard plumbagin was purchased from Sigma Aldrich Pvt. Ltd. Mumbai.

Instrumentation

Chromatographic separation was achieved on HPTLC plates using Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with 100 µL Hamilton syringe. For detection TLC scanner 3 with win CATS software (version 1.4.1) was used.

Preliminary studies

The microscopic and physico-chemical studies were carried out for *Shaddharana churna* as well as raw materials. Microscopic characters studied included tracheid, starch grains, cork, xylem vessel, and fibre. Physico-chemical studies included total ash, water soluble ash, acid insoluble ash, water soluble extractives, alcohol soluble extractives, and loss on drying[13,14].

Microscopic examination method[13,15]

The *Ayurvedic Churna* formulations were studied for their microscopic characteristics. Also, microscopy of powders of *Daruharidra*, *Katuka*, and *Chitraka* was done in order to confirm the presence of these drugs in the formulation. For determination of lignified characters, the *Churna* was boiled in chloral hydrate for 3-4 min and then, stained using phloroglucinol and hydrochloric acid solution. Starch grains were observed using iodine solution. All the observations were made under 10X eyepiece and 45X objective lens using compound microscope (motic).

Physico-chemical studies

Total ash value

About 2 g of accurately weighed formulation was placed in a suitable tared silica crucible. The material was incinerated by gradually increasing the heat, not exceeding 450 °C, until free from carbon. The crucible was cooled and weighed. Total ash value was calculated using the following formula:

Total ash value of the sample (% w/w) = 100 (C – A)/B

where A= weight of the empty crucible (g), B= weight of the formulation taken (g), C= weight of the crucible + ash (after complete incineration) (g).

Water–soluble and acid-insoluble ash

Total ash was boiled for 5 min with 25 mL of water. The insoluble matter was collected in a crucible and washed with hot water. It was then ignited at about 500 °C to constant weight. Water-soluble ash per g of air-dried material was calculated using the following formula:

Water – soluble ash value of the sample (%w/w) = 100 × D/B

Where B= weight of the formulation taken (g) and D= weight of insoluble matter (g). For acid-insoluble ash, the same procedure as that of water soluble ash was followed, using 25 mL of hydrochloric acid instead of water.

Alcohol-soluble and Water-Soluble extractive value

Accurately weighed 4 g of the formulation and transfered it to a 250 mL volumetric flask. Added 100 mL ethanol to the flask and kept aside for 24 h, shaking frequently (maceration). After 24 h, filtered till 25 mL of the filtrate was collected. Transferred the filtrate to a tared shallow and flat bottom disc. Evaporated to dryness on a water bath and completed the drying in oven at 105 °C. Cooled in a dessicator and weighed. Acid-soluble extractive value was calculated using the following formula:

Alcohol soluble extractive value of the sample (% w/w) = 100X

25 mL of alcoholic extract gives = X g of residue; 100 mL of alcoholic extract gives = 4X g of residue. Since, 4 g of air powdered formulation gives = 4X g of alcohol soluble residue; therefore, 100 g of powdered formulation drug gives = 100X g of the alcohol soluble residue.

Water soluble extractives were determined by following the same procedure as that of alcohol soluble extractives by using chloroform water instead of ethanol as a solvent.

Loss on drying

The percentage of active ingredients in the formulation under study was calculated on air dried basis. Presence of moisture may lead to decomposition of formulation such as chemical change or microbial contamination. Therefore it is important to determine and control the moisture content of the formulation. Accurately weighed 10 g of the formulation was placed in a tared and previously weighed evaporating dish. Then, dried in oven at 105 ºC for 5 h, cooled in a dessicator, and weighed. Drying was continued until two consecutive weighing did not differ by more than 0.25 %. The loss in weight is usually recorded as moisture.

HPTLC method development

Preparation of stock solutions and working standards

About 10 mg of each marker (Plumbagin, apocynin, and berberine) was transferred in 3 volumetric flasks of 10 mL and the volume was then made up with methanol to obtain solutions of 1000 parts per million (ppm). These were used as stock solutions and subsequent dilutions were made to obtaine working standards of 100 and 10 ppm.

Preparation of in-house formulation

In-house formulation was prepared by mixing all the 6 powders in equal proportions, according to the procedure given in *Ashtangasangraham*[4] (*Kushtachikitsa adhyaya*) to obtain a uniform *churna* preparation.

Extraction of plant constituents from formulations

Each formulation (25 g) was subjected to soxhlet extraction using chloroform as extracting solvent (250 mL). Soxhlet extraction was continued for 2 h. Prolonged times of extraction causes degradation of plumbagin[16]**.** Further, excess of solvent was evaporated using rotary evaporator. Dried extract (10 mg) was weighed and transferred to a 10 mL volumetric flask and the volume was then made up using methanol. These extracts were used later for quantification of markers.

Optimized Chromatographic conditions

The mobile phase optimized for separation was toluene: ethyl acetate: methanol: formic acid (5: 2.7: 1: 1 v/v/v/v). A saturation time of 20 min was maintained. Samples, each of 10 µL were applied, having 6 mm width, distanced 10 mm apart on HPTLC plates pre-coated with silica gel $60F_{254}$ (10 cm x 10 cm with 200 µm thickness). Linear ascending development with migration distance of 80 mm was carried out in Camag twin trough chamber, at room temperature (25±2 °C).

Scanning was performed in UV range, with slit dimension set to 5 x 0.30 mm, at a scanning speed of 100 mm/sec. After completion of scanning, the R_f values, peak areas and spectra of all the 3 markers

were recorded. A wavelength that is isoabsorptive for plumbagin and apocynin, and at which berberine shows significant absorbance was selected (270 nm) (Fig. 2). The R_f value obtained for berberine, apocynin, and plumbagin was 0.27±0.2, 0.56±0.2, and 0.75±0.2, respectively (Fig. 3).

HPTLC method validation[17]

HPTLC method validation was carried out according to ICH guidelines Q2 (R1). Following parameters were considered while performing the validation.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of an analyte in the sample. Linearity was evaluated by analysing the plot of area as a function of concentration of analyte. Test results were evaluated by calculating of regression coefficient (r^2).

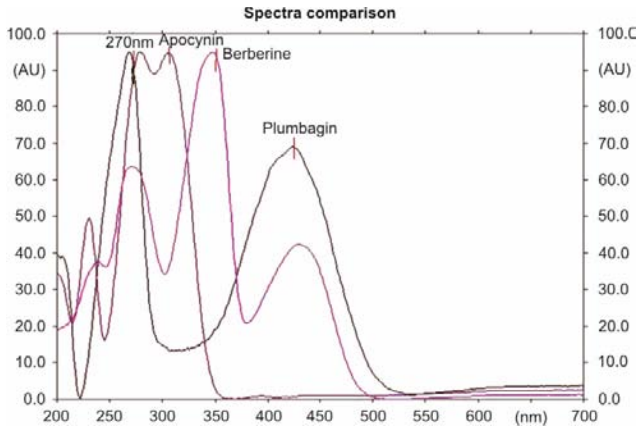

Fig. 2- Selection of analytical wavelegth

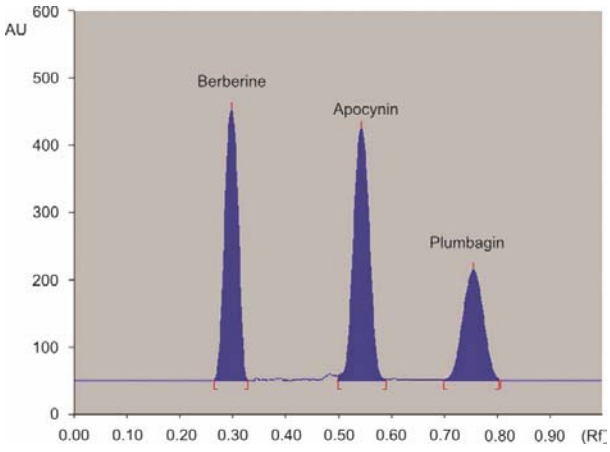

Fig. 3-Densitogram of the markers at optimized chromatographic conditions

Quantification of markers from extracts of the formulations

The amount of berberine, apocynin and plumbagin present in *Shaddharana churna* was calculated using the linear regression analysis. Quantification of the markers was done by performing HPTLC analysis of the extracts according to the developed method. Area obtained for each of the marker from each formulation was extrapolated on the respective calibration curve of that marker. This analysis was performed in triplicate. The results obtained were further used for the recovery experiments.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. It is performed in order to ensure the identification, purity testing, and quantitation of marker compound from the *Ayurvedic* formulation under study. Specificity was confirmed by comparing the R_f value and UV spectra of the standards with the component obtained in chromatogram of extract of different *Shaddharana churna* formulations.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found.

Recovery of berberine, apocynin and plumbagin from each of the 3 formulations was checked by spiking a known quantity of standards (80, 100, and 120 % of the quantified amount) to the test samples in triplicate using HPTLC. This way, accuracy was performed and calculated for nine determinations over a specified range and the mean recovery was calculated.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Three replicates of quality control (QC) samples of berberine, apocynin, and plumbagin at 3 different concentration levels i.e. low quality control (LQC), mid quality control (MQC), and high quality control (HQC) were analyzed on 3 different days (inter-day precision) and at 3 different times on a same day (intra-day precision).

Limit of detection (LOD)

The LOD of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Limit of quantitation (LOQ)

The LOQ of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. It is a parameter of quantitative assays for low levels of compounds (markers) in sample matrices (extracts) and is used particularly for the determination of interfering compounds. The LOD and LOQ are expressed as:

$LOD = 3.3\ \sigma/S$
$LOQ = 10\ \sigma/S$

Where, σ = the standard deviation of the response, S = the slope of the calibration curve. The slope S and standard deviation σ were estimated from the calibration curve of the individual marker compound. Considering numerous chemical constituents present in one herb, LOQ becomes an important parameter while analysing ayurvedic and herbal preparations.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was evaluated by making deliberate changes in the mobile phase composition and saturation time (±5 min).

Two concentrations of each of the marker were analyzed in triplicate in order to ensure that the method is robust.

Results

Microscopic characters

Identification characters of all the components are as follows: *Ativisha* (parenchymatous cells with starch, calcium oxalate crystals, sclereides in group, xylem with reticulate and spiral thickening); *Chitraka* (cubical or rectangular dark brown cells, starch containing cells, cells with yellow content, sclereids, fragments of vessels, parenchyma with thick wall, long fibres); *Daruharidra* (fragments of cork cells, fibres, stone cells single or groups, xylem vessels with spiral thickening, phloem ray cells, gland cells); *Katuka* (thin walled parenchyma, thick walled parenchyma, starch grains simple round to oval, fibres, pitted vessels); *Indravaya* (thin walled parenchyma with starch, thick walled parenchyma, cells with oil globules, stone cells, fibres, xylem with reticulate , spiral thickening); *Patha* (small and large starch grains, parenchyma with thin as well as thick wall, xylem with reticulate thickening, fibres). The microscopic characters observed in *Shaddharana Churna* are shown in plate 1.

Physico-chemical analysis

The results for physico-chemical analysis are summarized in Table 1. It was found that highest amount of total ash, water soluble extractives, and alcohol soluble extractives were 5.61, 29.00, and 25.00 % w/w, respectively were yielded by the in-house formulation.

Table 1- Results of physical evaluation of *Shaddharana churna*

Parameters	*Shaddharana churna*		
	M1	M2	In-house formulation
Total ash (% w/w)	10.89	12.19	25.61
Water soluble ash (% w/w)	5.94	4.87	5.94
Acid insoluble ash (% w/w)	4.95	2.92	1.47
Water soluble extractives (% w/w)	21.00	10.00	29.00
Alcohol soluble extractives (% w/w)	22.00	12.00	25.00
Loss on drying (% w/w)	3.26	3.82	3.58

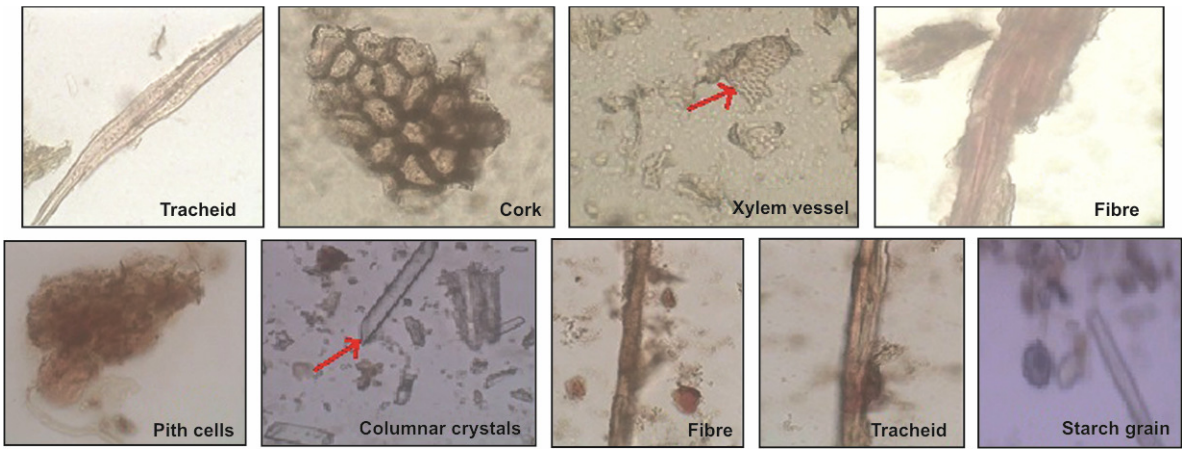

Plate 1-Microscopic characters observed in *Shaddharana churna*

Table 2 - Linear regression data for calibration curves of plumbagin, apocynin and berberine

Parameter	Plumbagin	Apocynin	Berberine
Linearity Range (ng)	200–800	25–175	200–700
Equation of regression line	y = 8.195x + 367.9	y = 24.62x + 417.3	y = 9.012x + 2122
Coefficient of correlation (r^2)	0.995	0.997	0.996
Slope	8.195	24.62	9.012
Intercept	367.9	417.3	2122

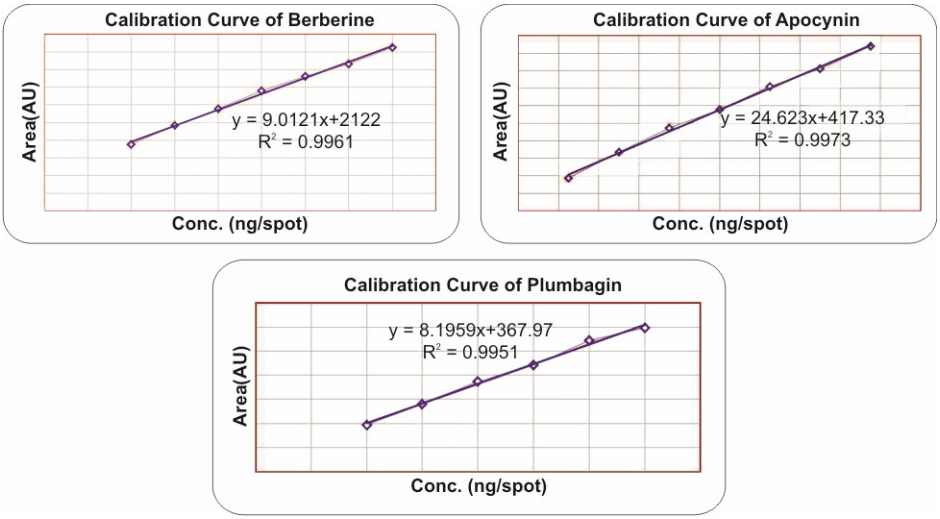

Fig. 4-Calibration curves of markers

Table 3- Quantification of markers

Markers	% w/w content of markers in *Shaddharana churna*		
	M2	M1	In-house formulation
Berberine	0.9163	0.2259	0.5657
Apocynin	0.0895	0.0456	0.0370
Plumbagin	0.6440	0.6220	0.4768

HPTLC profile

Linearity

Plumbagin, apocynin, and berberine showed linear response in concentration range of 200-800, 25-175, and 200-800 ng/spot, respectively. Interpretation of regression line validated the linearity (Table 2, Fig. 4).

Quantification of markers

The quantity of berberine, apocynin, and plumbagin were calculated using the calibration curves of the individual markers. The results are summarized in Table 3. It was observed that marketed formulation (M2) contained highest quantity of all the three markers, berberine 0.9136 % w/w, apocynin 0.0895 % w/w, and plumbagin 0.6440 % w/w.

Specificity

Specificity was confirmed by comparing the R_f value (Fig. 5) and UV spectra of the standards with the component obtained in chromatogram of extract of different *Shaddharana churna* formulations (Fig. 6 a-c).

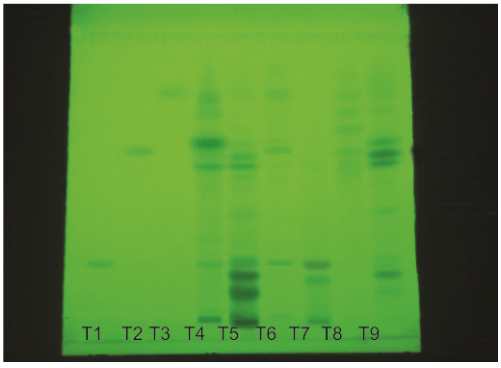

Fig. 5-Comparative HPTLC fingerprinting profile of extracts of *Shaddharana churna* formulations at 254 nm

Accuracy

The % recovery of all the 3 markers was found to be between 100 – 120 % (Table 4-6). Therefore, the method was found to be accurate.

Precision

The statistical analysis of the results proved that % relative standard deviation (RSD) of the peak areas obtained was less than 2 % (Table 7), hence, the developed method was found to be precise.

LOD and LOQ

The results for LOD and LOQ are summarized in Table 8. Plumbagin was found to be having the highest LOD and LOQ of 45.51 and 137.91 ng/spot,

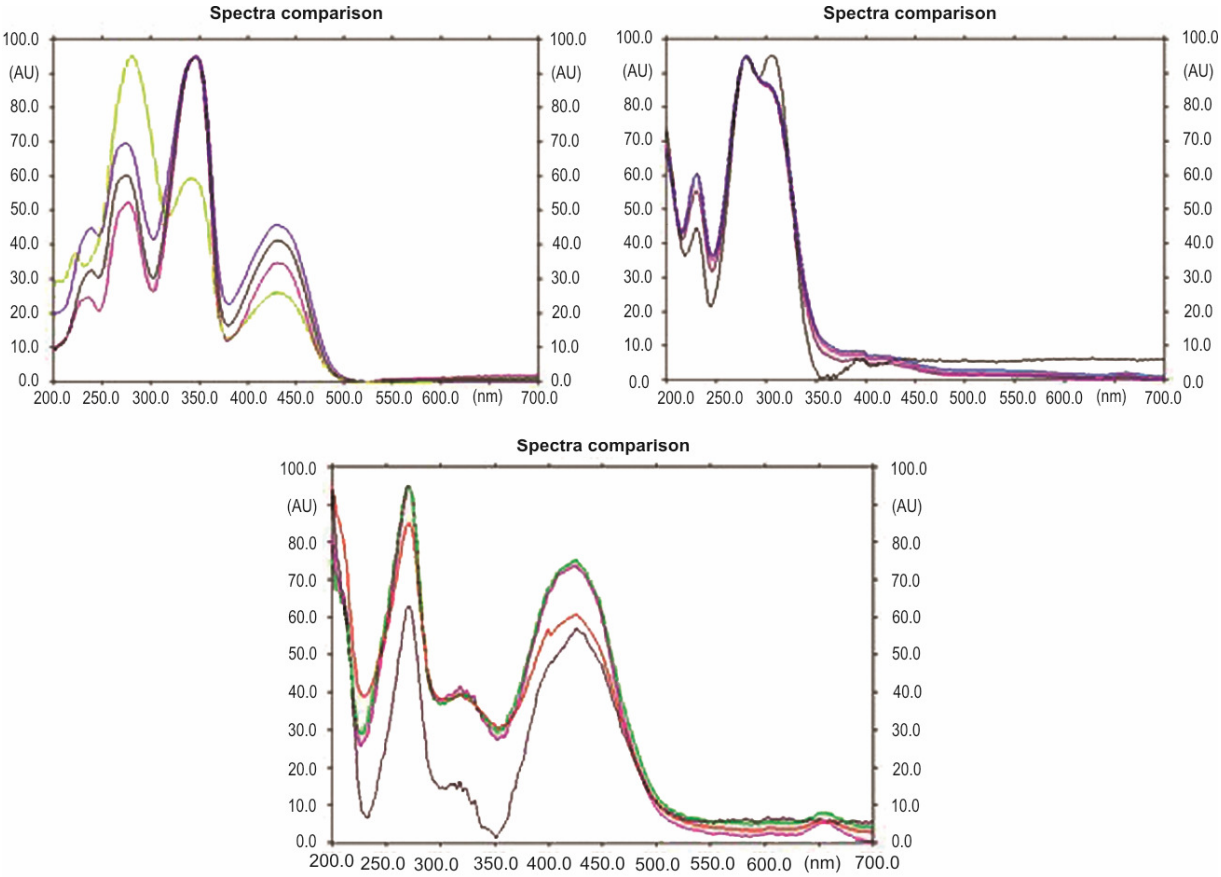

Fig. 6-Spectral overlain of markers and extract of marketed and in-house formulations *Shaddharana Churna*

Table 4- Accuracy table for berberine

Shaddharana churna	Level of recovery	Theoretical content of marker in ng	Amount of marker recovered in ng	% Recovery	Average % recovery
M2	80	896.4	904.7	100.9	
	100	996.0	983.2	98.7	99.2
	120	1095.6	1069.6	98.0	
M1	80	329.0	326.6	99.2	
	100	366.0	365.9	99.9	99.7
	120	402.0	395.9	98.4	
In-house formulation	80	565.2	552.2	98.0	
	100	628.0	621.7	99.0	98.8
	120	690.8	688.0	99.5	

Table 5- Accuracy table for apocynin

Shaddharana churna	Level of recovery	Theoretical content of marker in ng	Amount of marker recovered in ng	% Recovery	Average % recovery
M2	80	87.6	88.6	98.8	
	100	97.2	98.2	100.9	100.5
	120	107.1	108.9	101.7	
M1	80	66.6	65.6	98.4	
	100	74.0	73.3	99.0	99.6
	120	82.0	83.3	101.5	
In-house Formulation	80	33.3	33.7	100.9	
	100	37.0	36.9	99.7	100.6
	120	40.8	41.3	101.2	

Table 6- Accuracy table for plumbagin

Shaddharana churna	Level of recovery	Theoretical content of marker in ng	Amount of marker recovered in ng	% Recovery	Average % recovery
M2	80	630.0	634.5	101.1	
	100	700.0	699.6	100.0	100.3
	120	770.0	769.4	99.7	
M1	80	907.3	900.0	99.2	
	100	1008.0	983.6	97.6	98.9
	120	1108.8	1110.8	100.1	
In-house	80	531.0	523.3	98.5	
Formulation	100	590.0	577.7	98.0	98.6
	120	649.0	645.3	99.4	

Table 7- Results of analysis of precision for all the three marker compounds

Standard	Conc. (ng/spot)	Intra-Day Precision		Inter-Day Precision	
		SD	%RSD	SD	%RSD
Plumbagin	200 [LQC]	43.5	1.9	14.1	0.6
	400 [MQC]	79.1	1.8	49.4	1.1
	600 [HQC]	91.0	1.5	101.1	1.7
Berberine	300 [LQC]	54.3	1.1	41.1	0.8
	500 [MQC]	105.0	1.5	94.5	1.3
	700 [HQC]	123.0	1.5	134.1	1.6
Apocynin	50 [LQC]	38.10	2.0	31.4	2.0
	125 [MQC]	45.84	1.1	37.3	1.0
	200 [HQC]	11.88	0.2	68.9	1.3

Table 8 - LOD and LOQ of the marker compounds

Parameter	Berberine	Apocynin	Plumbagin
LOD in ng/spot	32.12	4.96	45.51
LOQ in ng/spot	97.33	15.05	137.91

Table 9- Results of analysis of robustness of the method for all the three marker compounds

Parameter	%RSD					
	Berberine		Apocynin		Plumbagin	
	300 ng/spot	500 ng/spot	75 ng/spot	150 ng/spot	400 ng/spot	600 ng/spot
Mobile Phase Composition						
T: E.A.: M: F[1] 5: 3: 1: 0.7[2]	0.62	0.87	0.81	0.45	1.64	1.30
T: E.A.: M: F 4.7: 2.7: 1.3:1	1.91	0.57	1.86	1.14	0.75	1.50
Saturation Time						
25 min	1.45	0.74	1.03	0.84	0.98	0.54
15 min	0.10	0.65	1.94	1.23	1.93	0.95

[1]T– Toluene, E.A.– Ethyl acetate, M– Methanol, F – Formic acid, [2]Unit: volume (v)/ v/ v/ v

respectively, whereas apocynin had the lowest LOD and LOQ of 4.96 and 15.05 ng/spot, respectively.

Robustness

The % RSD value for all the results is less than 2 % (Table 9). Hence, the method was found to be robust.

Conclusion

A sensitive HPTLC method was developed for simultaneous estimation of berberine, apocynin, and plumbagin from *Shaddharana churna*. The developed HPTLC method was found to be simple, precise, accurate, specific, and reproducible for standardization of *Shaddharana churna*. This method will ensure purity and efficacy of the formulations. Therefore, it can be used for quality evaluation and standardization of formulations containing berberine, apocynin, and plumbagin alone or in combination, as marker compounds.

Acknowledgement

The authors are thankful to Dr KS Laddha for gift sample of berberine.

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