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High performance thin layer chromatography qualitative densitometry as a sensitive method to assess shelf life of polyherbal formulations: A study on *Hutabhugadi Churna*

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Abstract

Introduction: Measuring chemical stability of polyherbal formulations is very challenging due to diversity in phytochemical composition. As there are no comprehensive guidelines for stability testing of herbal products, there is a need for a sensitive tool to detect how the quality of herbal products varies with time under the influence of environmental conditions. **Aims:** To validate the employability of high-performance thin layer chromatography (HPTLC) for real-time stability of *Hutabhugadi Churna* (HC). **Materials and Methods:** The chromatograms were developed using toluene/ethyl acetate/formic acid (10:5:1) and ethyl acetate/formic acid (10:1) as a mobile phase for chloroform and ethanolic extract, respectively. The plates were scanned under 254, 366, 540 (pre-derivatization) and 540 nm (post-derivatization). Samples were analyzed immediately after preparation and after 3rd and 6th months of storage. Alteration of fingerprint profiles from the initial pattern, in terms of number of peaks, was employed as diagnostic tools. Percentage variation in composition at given period was calculated. **Results:** HC is found to be stable at room temperature up to 1.3 months using the method of calculation of 10% degradation period employing slope and intercept values for the initial, 3rd and 6th months' deviation in number of bands. The data obtained were subjected to regression analysis in context to number of bands obtained. The curve was found to be linear with R^2 value of 0.89–0.96 supported by their tolerance range of 0.04–0.11. **Conclusion:** The proposed model is a new logic with prospects to become working method for stability assessment of polyherbal formulations under controlled conditions.

Key words: Densitometry, high-performance thin layer chromatography, real-time stability, shelf life

Introduction

Herbal drugs have been used since ancient times as medicines for the treatment of a range of diseases. Medicinal plants have played a key role in world health.[1] At present, India contributes <1% to the global herbal market. However, it is fast emerging as a key supplier of medicinal plant formulations across the globe.[2]

The most important challenge faced by herbal formulations arises from lack of complete evaluation of its constituents, due to their complex nature. During production, from cultivation of the plant to the finished product, a lot of changes take place in the chemical structure. Due to the complex composition of herbal preparations, an analysis is mostly done by running high performance liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC),

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or by quantitative determinations using ultraviolet-visible spectroscopy or combinations of these. HPLC and GC methods have the benefit that a specific fingerprint chromatogram for identification and purity testing, as well as the detection of single compounds for assay is possible during one analysis. These specific methods are now-a-days generally expected by the authorities. However especially in the case of a combined product with two, three or even more active ingredients, a specific determination and quantification of each drug preparation is often impossible.[3]

Evaluation of these constituents is necessary to ensure quality, purity and stability of the herbal product. Stability study provides evidence on how quality of a drug substance or product varies with time under influence of variety of environmental factors such as, temperature, humidity, light, and storage conditions. So, stability study is important in the evaluation of quality of the product. Stability testing should be conducted on the dosage form packed in the container closure system proposed for marketing. With the help of modern analytical techniques, such as spectrophotometry, HPLC, high performance TLC (HPTLC) and by employing proper guidelines it is possible to generate a sound stability data of herbal products and predict their shelf life, which will help in improving global acceptability of herbal products.[4]

There are no specific guidelines available for stability/shelf life estimation of the pure Ayurvedic formulations from any Government organization except a gazette notification issued by Government of India on October 20, 2009 with slight modification in the earlier draft notification issued on November 26, 2005.[5,6]

HPTLC has wide application as an analytical tool due to its simplicity, minimum sample-cleanup requirement, and ability to analyze a number of samples simultaneously. In the present investigation, real-time stability study was conducted for *Hutabhugadi Churna* (HC) a herbo-mineral formulation used in the treatment of *Agnimandya* (digestive impairment), *Pandu* (anemia), *Sopha* (oedema) and *Arsa* (piles) in Ayurveda.[7] The HPTLC method is meaningful as a small variation in the chemical composition would alter the fingerprint profile giving a diagnosis of decomposition.

Materials and Methods

Formulation and packing

HC was prepared with ingredients as per Ayurvedic Formulary of India (AFI) [Table 1].[7] All the ingredients except *Saindhava Lavana* were washed thoroughly to have no microbial load.[8] The washed and dried raw drugs were finely powdered. *Saindhava Lavana* was roasted in a stainless steel pan on low flame until free from moisture and then powdered. The individual raw drug powders were passed separately through sieve number 44 followed by 85. Each ingredient was weighed separately and mixed together in the proportion specified and passed through sieve number 44 to obtain a homogenous blend.[9] The formulation was compliant to quality standards reported earlier.[10]

Packing and storage

The formulation was packed in an air-tight plastic container with sealing of aluminum foil. Six bottles containing 50 g each of the formulation were kept for real time stability study at an interval of 0, 3 and 6 months. The studies were carried out at temperature: 25°C ± 2 and relative humidity: 60% ±5.

Chemicals and reagents

All chemicals used in the experiment were of analytical grade and of Merck.

Extract preparation

HC (5 g) was successively extracted with 150 ml of chloroform and ethanol using Soxhlet apparatus. The filtrate was concentrated to dryness and dried residue was dissolved in 5 ml of respective solvents (chloroform and ethanol) in a 10 ml volumetric flask, and made up to 10 ml with same solvent. Four microliters from the above solution was used as application volume for both the extracts.

Chromatographic study

HPTLC of both chloroform and ethanolic extract of HC was carried out using optimized mobile phase during initial days of preparation, 3rd and 6th months.

Mobile phase

Different solvent systems were tried for the chromatographic separation of both chloroform and ethanolic extracts. The following solvent systems gave optimum separation and hence used for the HPTLC study: toluene:ethyl acetate:formic acid (10:5:1, v/v) for chloroform extract and Ethyl acetate: formic acid (10:1, v/v) for ethanolic extract.

Development of chromatograms

Chloroform and ethanolic extract of HC were applied separately on aluminum plates pre-coated with silica gel 60 F_{254} of 0.2 mm thickness (Merck, Germany) using LINOMAT 5 applicator (CAMAG). The plates were developed in CAMAG twin trough chamber previously saturated with mobile phase. After photodocumentation using CAMAG visualizing chamber and densitometric scan using CAMAG Scanner 4 at wavelengths of 254 nm, 366 nm and 540 nm the plates were derivatized by spraying vanillin-sulfuric acid followed by heating at 105°C until the bands appeared.[11-13] The R_f values and densitometric

Table 1: Formulation composition

Ingredient	Botanical name	Part	Proportion
Hutabhuga (Citraka API)	*Plumbago zeylanica* Linn.	Root	1 part
Ajamoda (Ajamoda API)	*Apium leptophyllum* (Pers.) F.V.M. ex Benth.	Fruit	1 part
Saindhava lavana	Rock salt (Halite)	-	1 part
Magadha (Pippali API)	*Piper longum* Linn.	Fruit	1 part
Marica API	Piper nigrum Linn.	Fruit	1 part
Pathya (Haritaki) API	Terminalia chebula Retz.	Pericarp	5 parts

data were documented using winCATS Planar Chromatography Manager version 1.4.6 (CAMAG Muttenz, Switzerland).

Application of three extracts of HC (initial, 3rd and 6th) could not be performed on same TLC plate as extract of HC initial will not be same as fresh extract HC 3rd month (as the environmental conditions of HC will be totally different from extract of HC). TLC plates, photo-documented at three durations separately were combined after completion of 6th month's study using Adobe Photoshop® for side by side comparison of finger prints to observe the difference, if any.

Real time stability assay

The change in HPTLC profile was observed for 6 months at an interval of 0 (initial), 3 and 6 months. Number of months when 10% degradation occurred was calculated using formula = ([0 Month Assay value – [[0 Month Assay value × 10]/100]] – Intercept)/Slope.[14]

Results

Marked variations in TLC plate were observed in both chloroform and ethanol extracts of HC [Figure 1]. Chloroform extract of HC showed 11 bands in initial month, 7 bands in 3rd and 6 bands in 6th month at 254 nm; at 366 nm it has shown 9, 8 and 4 bands in initial, 3rd and 6th months, respectively; under 540 nm before derivatization it has shown 3 and 2 bands at initial and 3rd months, whereas no bands are observed in 6th month; at 540 nm after derivatization it has shown 11, 9 and 8 bands in initial, 3rd and 6th months [Table 2 and Figure 2]. Ethanolic extract of HC at 254 nm showed 9, 7 and 6 bands during initial, 3rd and 6th months, respectively; at 366 nm there were 9, 7 and 3 bands during initial, 3rd and 6th months, respectively; at 540 nm there were 8, 6 and 4 bands in initial, 3rd and 6th months, respectively and at 620 it has shown 7, 9 and 11 bands in initial, 3rd and 6th months, respectively [Table 3 and Figure 3]. There was a gradual decrease in the number of bands in 3rd and 6th months when compared to initial. In ethanolic extract, at 540 nm after derivatization, there was increase in number of bands in older samples than recorded initially. Numbers of bands obtained at different intervals at different wavelengths of chloroform and ethanol extract were subjected to regression analysis. The curve was found to be linear with R^2 value of 0.89-0.96 supported by their tolerance range of 0.04-0.11. The method of calculation of average of 10% degradation period employing slope and intercept values for the initial, 3rd and 6th months' deviation in number of bands at different wavelengths of light was employed. For chloroform extract 10% deviation in the number spots was observed during 0.72–1.87 months (standard deviation [SD] =0.5605) at different wavelengths. But of ethanol extract it was in the range of 1.05-1.47 months with SD of 0.1746 only. When average of 10% deviation was considered for both extracts fingerprinted at different wavelengths HC was found to be stable at room temperature up to 1.2675 months (SD = 0.385922) [Table 4 and Graph 1].

Discussion

In chloroform extract, at 254 nm the spot at R_f 0.62 seen during 3 months with 39.40% area was not there initially and after 6 months. At 366 nm spot at R_f 0.85 showed 1.68% initially, after 3 months the area raised to 30.68%, but 6 months it was not seen instead it revealed a spot at R_f 0.87 with area of 88.40%. In ethanol extract, at 254 nm the spot at R_f 0.45 was observed in initial sample with area of 9.76%, but it was absent during 3 months, during 6 months the spot reappeared as a major spot having area of 51.70%. All these changes may be attributed to chemical interaction taking place in between the chemical components present in the polyherbal medicines resulting in degradation of composition which was there when the medicine

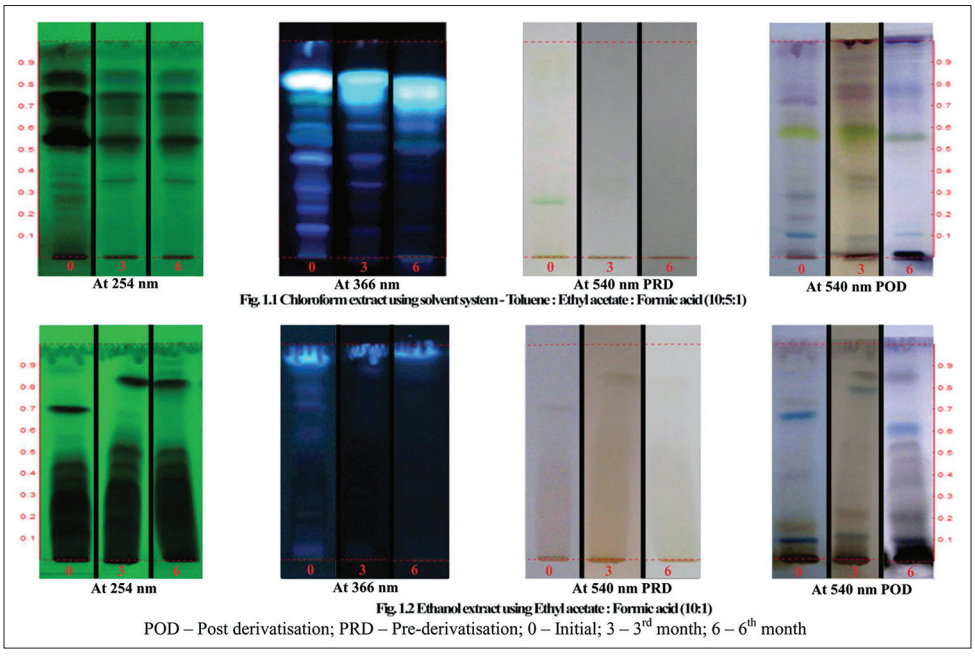


Figure 1: Photodocumentation of extracts of *Hutabhugadi churna* at different intervals

Table 2: Densitometric scan of chloroform extract of *Hutabhugadi Churna*

	At 254 nm						At 366 nm					
	Initial		3rd month		6th month		Initial		3rd month		6th month	
	R_f	Area %	R_f	Area %	R_f	Area %	R_f	Area %	R_f	Area %	R_f	Area %
	0.04	13.22	-	-	0.04	0.35	-	-	-	-	0.01	0.51
	0.16	2.03	-	-	-	-	0.05	3.14	-	-	-	-
	-	-	0.22	0.26	-	-	0.19	2.63	-	-	-	-
	0.24	1.89	-	-	-	-	0.29	2.05	-	-	-	-
	0.29	2.19	0.29	1.23	-	-	0.34	1.46	0.35	1.46	-	-
	-	-	-	-	0.31	0.4	0.39	2.77	0.38	1.64	-	-
	0.34	6.01	-	-	-	-	-	-	0.41	1.37	-	-
	0.41	3.27	0.40	8.48	-	-	-	-	0.47	0.99	-	-
	0.47	1.49	-	-	-	-	-	-	-	-	0.50	3.44
	-	-	-	-	0.49	1.14	0.55	2.21	-	-	-	-
	-	-	-	-	0.59	36.31	-	-	-	-	0.58	7.65
	-	-	0.62	39.40	-	-	-	-	0.61	1.23	-	-
	0.65	37.06	-	-	-	-	0.65	4.85	-	-	-	-
	0.78	6.30	0.79	20.87	0.79	44.24	-	-	0.68	0.44	-	-
	-	-	0.84	15.44	-	-	0.85	1.68	0.85	30.68	-	-
	0.87	20.76	-	-	0.88	17.52	-	-	-	-	0.87	88.40
	-	-	0.93	14.31	-	-			0.92	62.19	-	-
	0.95	5.78	-	-	-	-	0.95	79.22	-	-	-	-
Number of peaks	11		7		6		9		8		4	
	At 540 nm						At 620 nm					
	0.03	57.74	-	-	-	-	-	-	0.03	11.62	0.02	7.95
	0.32	32.86	0.33	31.61	-	-	0.05	19.88	-	-	-	-
	-	-	0.39	68.39	-	-	0.11	2.52	0.12	4.55	0.13	2.83
	0.61	9.40	-	-	-	-	0.16	20.64	-	-		
							-	-	0.19	1.33	0.18	1.64
							0.23	4.18	-	-		
							0.25	4.57	-	-		
							0.36	16.53	-	-	-	-
							-	-	0.38	8.66	0.39	1.97
							-	-	0.42	12.54	-	-
							0.44	2.32	-	-	-	-
							-	-	-	-	0.55	7.11
							0.56	1.44	-	-	-	-
							-	-	-	-	0.63	33.46
							-	-	0.66	36.04	-	-
							0.70	14.90	-	-	-	-
							-	-	-	-	0.78	8.00
							0.85	8.66	0.85	12.00	-	-
							-	-	-	-	0.86	37.04
							-	-	0.88	10.17	-	-
							0.91	4.36	-	-	-	-
							-	-	0.96	3.10	-	-
Number of peaks	3		2		-		11		9		8	

was prepared afresh. The HPTLC study of the samples was carried out separately as and when the batch reached the specified maturity. The samples were fingerprinted by HPTLC more than once during the particular analysis period (initial, 3 and 6 months) in the experimental conditions of the laboratory to find out the consistency of the results. The fingerprint of HC did not vary significantly in the laboratory conditions. As HPTLC fingerprint significantly sensitive to uncontrollable laboratory conditions like relative humidity, temperature etc., there may be slightest variation. All other conditions followed in the fingerprinting were controlled including the person who handled it.

Stability testing is necessary to ensure that the product is of satisfactory quality throughout its storage period. The expiry dates are explained in detail in ancient Ayurvedic text

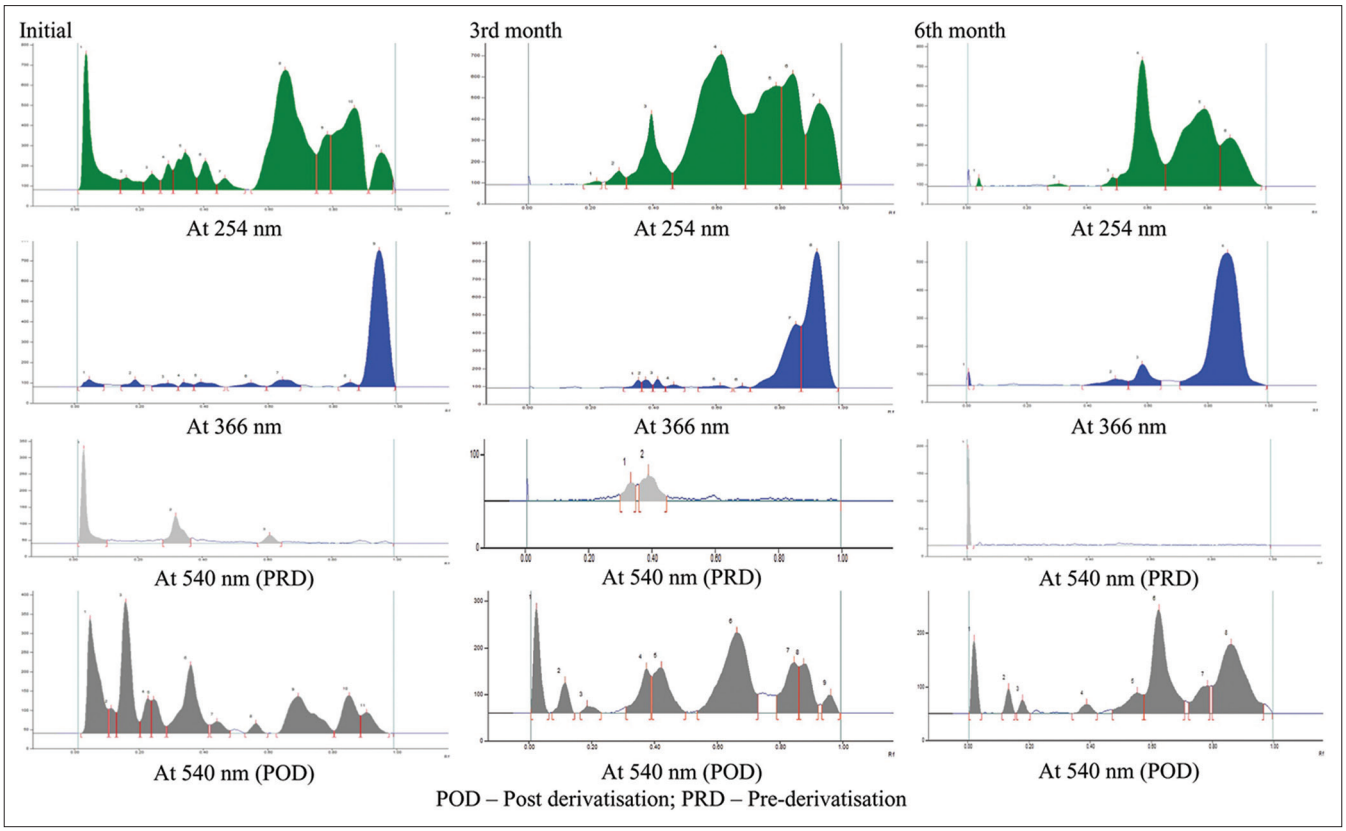


Figure 2: Densitometric scan of chloroform extract of *Hutabhugadi churna* at different intervals

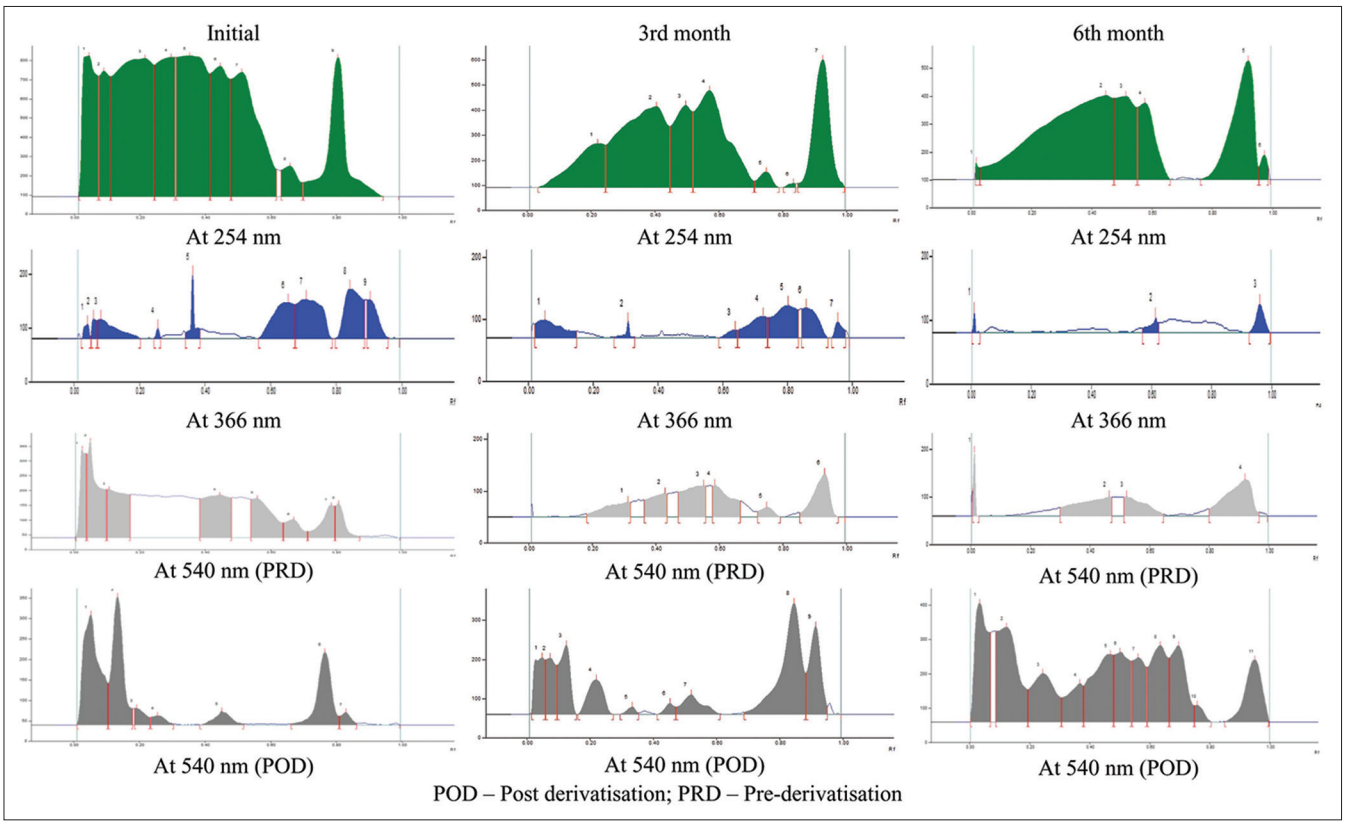


Figure 3: Densitometric scan of ethanol extract of *Hutabhugadi churna* at different intervals

Table 3: Densitometric scan of ethanolic extract of *Hutabhugadi Churna*

	At 254 nm						At 366 nm					
	Initial		3rd month		6th month		Initial		3rd month		6th month	
	Maximum position	Area %	Maximum position	Area %	Maximum position	Area %	Maximum position	Area %	Maximum position	Area %	Maximum position	Area %
	-	-	-	-	0.02	0.48	-	-	-	-	0.01	9.64
	0.05	8.48	-	-	-	-	0.04	1.86	0.05	19.19	-	-
	0.09	5.51	-	-	-	-	0.06	2.41	-	-	-	-
	0.22	21.55	0.22	11.68	-	-	0.08	9.30	-	-	-	-
	0.30	10.44	-	-	-	-	0.26	0.83	-	-	-	-
	0.35	17.45	-	-	-	-	-	-	0.31	2.81	-	-
	-	-	0.41	31.00	-	-	0.36	5.67	-	-	-	-
	0.45	9.76	-	-	0.45	51.70	-	-	-	-	0.61	30.58
	0.51	14.33	0.50	12.50	0.51	13.82	0.65	20.08	0.64	3.38	-	-
	-	-	0.57	25.34	0.58	11.07	0.71	25.98	-	-	-	-
	0.66	2.02	-	-	-	-	-	-	0.73	17.72	-	-
	-	-	0.75	1.70	-	-	-	-	0.80	31.31	-	-
	0.81	10.45	-	-	-	-	0.84	23.14	-	-	-	-
			0.83	0.26	-	-	-	-	0.86	20.47	-	-
			0.93	17.53	0.92	21.52	0.90	10.73	-	-	-	-
			-	-	0.97	1.41	-	-	0.96	5.10	0.96	59.79
Number of peaks	9		7		6		9		7		3	
	At 540 nm						At 620 nm					
	-	-	-	-	0.01	7.01	-	-	-	-	0.03	12.68
	0.03	9.50	-	-	-	-	0.05	36.57	0.05	9.31	-	-
	0.05	19.03	-	-	-	-	-	-	0.07	8.86	-	-
	0.11	16.93	-	-	-	-	0.13	29.31	0.13	12.18	0.12	16.42
	-	-	0.31	13.27	-	-	0.19	3.70	-	-	-	-
	-	-	0.43	14.40	-	-	-	-	0.22	8.87	-	-
	0.45	20.77	-	-	0.46	29.86	-	-	-	-	0.24	9.15
	-	-	-	-	0.52	19.13	0.26	2.61	-	-	-	-
	0.56	15.04	0.55	24.49	-	-	-	-	0.33	1.0	-	-
	-	-	0.59	19.60	-	-	-	-	-	-	0.37	4.96
	0.67	5.25	-	-	-	-	0.45	4.56	0.45	1.66	-	-
	-	-	0.75	4.36	-	-	-	-	-	-	0.47	11.62
	0.79	7.83	-	-	-	-	-	-	-	-	0.50	8.38
	0.81	5.66	-	-	-	-	-	-	0.52	6.43	-	-
	-	-	0.93	23.88	0.92	44.00	-	-	-	-	0.56	6.69
							-	-	-	-	0.64	10.67
							-	-	-	-	0.70	9.66
							0.77	20.68	-	-	0.76	1.20
							0.83	2.56	-	-	-	-
							-	-	0.85	35.09	-	-
							-	-	0.91	16.54	-	-
							-	-	-	-	0.95	8.60
Number of peaks	8		6		4		7		9		11	

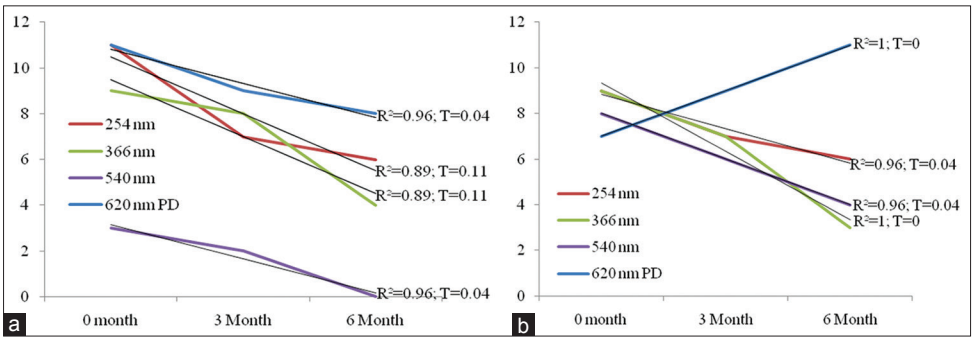
books. Taking that into consideration, the Government of India has established the shelf life period of the Ayurvedic medicines. According to that, shelf life of *Churna* is 2 years (once the container is opened, it should be consumed within 2-4 months).[15] Similar study employing variation in pharmacopoeial constants during the course of 6 months' evaluation revealed real time shelf life of HC to be 11.41 months by evaluation of many pharmacopoeial constants such as loss on drying at 105°, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive and water soluble extractive.[16]

The trend line showed down trend hence this work can be considered as prospective to become model for stability study. Trend line of bands of ethanolic extract, after derivatization with vanillin sulfuric acid, showed reverse trend between samples hence that counter acts the basic hypothesis that decrease in band reflects degradation of sample; increase in

Table 4: Stability of *Hutabhugadi churna* at different intervals and time for 10% degradation

Extract and scan wavelength	Number of spots in initial month	Number of spots in 3rd month	Number of spots in 6th month	Slope	Intercept	Results at 10% degradation	10% degradation (months)	Average (months)
Chloroform extract (nm)								
254	11	7	6	0.8333	10.5	9.9	0.72	1.2675
366	9	8	4	0.8333	9.5	8.1	1.68	
540	3	2	0	0.5	3.166	2.7	0.93	
620 (PD)	11	9	8	0.5	10.8333	9.9	1.87	
Ethanolic extract (nm)								
254	9	7	6	0.5	8.8333	8.1	1.47	
366	9	7	3	1	9.3333	8.1	1.23	
540	8	6	4	0.666	8	7.2	1.19	
620 (PD)	7	9	11	0.6667	7	6.3	1.05	

PD: Postderivatisation



Graph 1: Regression diagnostics of deviation in number of bands. (a) Chloroform extract; R^2: Regression; T: Tolerance. (b) Ethanolic extract; R^2: Regression; T: Tolerance

number of bands can also be considered as degradation by modulation in basic molecular content. The HPTLC method proposed in this study, being direct tracking of variation in phytochemical fingerprint by TLC pattern, is more sensitive to detect deterioration in the quality of polyherbal formulations.

Conclusion

From the current study, it is observed that as the time departs, the numbers of bands are reduced or increased from the initial value. The methodology can be employed as an effective analytical tool for stability check of traditional polyherbal medicines without employing any marker compound. These preliminary findings have some impact on the stability of the composition of the polyherbal formulations of Ayurveda. Advancement in this line may render HPTLC as a useful technology in shelf life assessment. The proposed model is a new logic with prospective to become working method for stability assessment of polyherbal formulations under controlled conditions, however validation following measures as repeatability, accuracy, precision, specificity, selectivity, etc., are essential before declaring it as the model for the purpose. Moreover, the findings are just based on real time observations, accelerated stability studies would also be necessary to draw a final conclusion.

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Conflicts of interest

There are no conflicts of interest.

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हिन्दी सारांश

हाई परफोर्मेन्स थिन लेयर क्रोमेटोग्राफी क्वालिटेटिव डेन्सिटोमेट्री का एक संवेदनशीलप्रक्रिया के रूप में हुतभुगादी चूर्ण की वास्तविक समय स्थिरता के मूल्यांकन का अध्ययन

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फायटोकेमिकल संरचना में विविधता की वजह से पॉलीहर्बल योगों के रासायनिक स्थिरता माप बहुत चुनौतीपूर्ण है। समय के साथ हर्बल उत्पादों की गुणवत्ता में पर्यावरण की स्थिति के प्रभाव से बदलाव आता है, इसे पता लगाने के लिए एक संवेदनशील विधि की जरूरत है। इस लिए हर्बल उत्पादों की स्थिरता के परीक्षण के लिए कोई व्यापक दिशानिर्देश जरूरी हैं। इस अध्ययन में अग्निमान्द्य, पांडु, शोफ और अर्श के उपचार में इस्तेमाल होने वाला एक हर्बो–मिनरल आयुर्वेदीक योग हुतभुगादी चूर्ण कि वास्तविक समय स्थिरता के लिए हाई परफोर्मेन्स थिन लेयर क्रोमेटोग्राफी (एचपीटीएलसी) कि उपयोगिता सिध्द करने का प्रयास किया गया है। यह विधि, अपघटन के निदान देने में सार्थक है क्योंकि रासायनिक संरचना में एक छोटे से बदलाव से फिंगरप्रिंट प्रोफ़ाइल में परिवर्तन होता है। इस प्रयोग के लिए क्लोरोफॉर्म और एथनोलिक एक्सट्रेक्ट को मोबाइल चरण क्रमश: टोल्यून/एथिल एसीटेट/फार्मिक एसिड (१०:५:१) और एथिल एसीटेट/फार्मिक एसिड (१०:१) में क्रोमेटोग्राम मे विकसित किया गया। प्लेटों को २५४, ३६६, ५४० एनएम (पूर्व डिराइवटाईझेशन) और ५४० एनएम (पश्चात डिराइवटाईझेशन) के तहत स्कैन किया गया। नमूने तैयार करने के बाद और भंडारण के ३ और ६ महीने के बाद तुरंत विश्लेषण किया गया। फिंगरप्रिंट प्रोफ़ाइल में प्रारंभिक पैटर्न से चोटियों के संख्या में परिवर्तन को नैदानिक विधि के रूप में नियुक्त किया गया है। चुना गया समय अवधि में संरचना में प्रतिशत भिन्नता गणना की गई है। प्रारंभिक, ३ और ६ महीने का विचलन के लिए बैंड की संख्या में १०% गिरावट की अवधि की गणना की विधि का उपयोग के बाद, ढलान और अवरोधन मूल्यों की उपयोगिता से हुतभुगादी चूर्ण १.३ महीने के लिए कमरे के तापमान पर स्थिर हो पाया है। प्राप्त आंकड़ों के बैंड संख्या का प्रतिगमन विश्लेषण किया गया है। प्रतिगमन वक्र के आर२ मूल्य ०.८९–०.९६ श्रृंखला के द्वारा समर्थित सहिष्णुता ०.०४–०.११ के साथ रैखिक हो पाया है। प्रस्तावित मॉडल नियंत्रित परिस्थितियों में पॉलीहर्बल योगों की स्थिरता के मूल्यांकन के लिए एक नया विधि बन सकता है।फ