

## SCIENTIFIC EVIDENCE FOR STABILITY EVALUATION AND SHELF-LIFE ESTIMATION OF VACHA CHURNA BY USING RP-HPLC

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### ABSTRACT

**Objective:** The concept of shelf life of classical Ayurvedic formulations is clearly defined in the classical literature of Ayurveda. Considering the Ayurveda development and recent gazette notification of 161-B, it is important to evaluate the stability of the Ayurvedic products based on scientific evidence. The study aims to evaluate the stability and estimate the shelf life of Vacha churna by using the marker Beta-asarone. The chemical marker of the Vacha churna has been identified as Beta-asarone.

**Methods:** A stability-indicating RP-HPLC method has been developed and validated for Beta-asarone. The method utilises a Stationary phase C<sub>18</sub> column and a mobile phase composed of acetonitrile and ammonium acetate buffer. Beta-asarone underwent various stress conditions like acid and base hydrolysis, oxidation, temperature and photolytic degradation. The Vacha churna has been stored in a stability chamber for both accelerated and long-term testing. The formulation was taken at specific time intervals and the marker content was analysed using RP-HPLC.

**Results:** Beta-asarone was eluted at 6.03 min. The linear regression equation was found to be  $y = 28211x - 1349.3$  with a  $R^2$  of 0.9991. Stress studies confirmed the absence of co-eluting peaks and degradants under various stress conditions. Based on the analysis of Beta-asarone marker content, the shelf life of Vacha churna was estimated to be 62.40 mo.

**Conclusion:** Data from RP-HPLC method and stability studies can be employed for routine commercial stability assessments. Due to the absence of a Pharmacopoeial RP-HPLC method, local Ayurvedic manufacturers can utilise the developed method. The investigated shelf life thus serves as a medium of chemical stability based on the scientific evidence.

**Keywords:** Vacha churna, Beta-asarone, Shelf life, Stability studies, Scientific evidence

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### INTRODUCTION

Vacha is considered as an important medicinal plant in the Ayurvedic medicine and it is scientifically referred as *Acorus calamus linn*. Vacha has been traditionally employed in the therapeutic management of epilepsy, schizophrenia, constipation, dyspepsia, otitis media, cough, asthma and also it has antioxidant, hypolipidemic, anthelmintic and antispasmodic properties. The major bioactive compounds of Vacha are alpha and beta-asarone and eugenol, they form the primary marker compounds for the chemical fingerprinting of Vacha churna [1-4].

Based on data provided by the World Health Organisation, it is estimated that approximately 70-80% of global population depend on nonconventional medicines, primarily derived from herbal sources, for their healthcare needs. Despite the extensive development of Ayurveda over centuries, there remain concerns regarding the lack of high-quality medicines, stability and safety [5]. So, there is an urgent need to establish quality control, standardisation and stability profiles for ayurvedic products through the application of advanced analytical techniques.

Stability testing investigates the impact of environmental variables such as temperature, humidity, light, microbiological parameters on the quality of drug substance or formulated product. This evaluation predicts the products shelf life, determines proper storage methods and suggests suitable labelling instructions. In addition, the data generated from stability testing plays an important role in obtaining regulatory approval of any product [6].

The AYUSH ministry has revised and released a gazette notification in August 2016, specifically Rule No. 161-B of the drugs and cosmetics Act, which pertains to the stability study of Ayurveda formulations. According to this notification, it is necessary to conduct stability studies

and evaluate shelf life. The guideline mandates that Ayurvedic formulations to have a specific shelf life or expiry date and that must be determined through real-time stability investigations of formulations listed in the Indian Ayurvedic Pharmacopoeia and supported by scientific data.

The shelf life of Churna formulations is stated as 2 y in the Gazette notification, but it lacks the scientific justification [7-10]. So, it is necessary to assess the shelf life of Churna formulations on the basis of empirical evidence. The implementation of a validated scientific technique and a stability assessment module will provide opportunities for evaluating the chemical stability of traditional formulations. Therefore, an analytical method must be developed to assess the stability and to estimate the shelf life of Vacha churna.

To date, there is a lack of available stability profiles for this formulation. Hence, the current study employed RP-HPLC to evaluate the chemical stability and expiry date of Vacha churna within specified storage parameters. The objective of the present research is to develop a stability-indicating assay method for the quantification of Beta-asarone using RP-HPLC and to conduct accelerated (40 °C±2 °C/75% RH±5% RH) and long-term (30 °C±2 °C/65% RH±5% RH) stability studies of Vacha churna as specified in the ICH Q1A(R2) guidelines for stability testing.

### MATERIALS AND METHODS

#### Chemicals and reagents

The marker Beta-asarone (purity>95 %) was obtained from Sigma-Aldrich, India. The experiment utilized HPLC grade acetonitrile and methanol procured from Finar chemicals, India. Vacha churna was purchased from the local Ayurvedic manufacturer. The buffer solution for the mobile phase was prepared using Milli Q water

obtained from Merck, India. All the chemicals or reagents utilized were of analytical reagent (AR) grade.

### Instrumentation

Electronic analytical weighing balance of Shimadzu (AY220) was used for weighing. Ultra-sonicator from LABMAN was used for the study. The UV-visible spectrophotometer of Shimadzu (UV 1800) make equipped with UV probe software was used for recording the UV spectrum. Shimadzu HPLC system equipped with the System controller: SCL-10A VP, Photodiode array detector (PDA): SPD-M10A VP, auto injector: SIL-20AC HT, pump: LC-20AD, Column oven: CTO-10AS VP and LC Solution software was utilized for the data acquisition and interpretation. Thermal stress studies were carried out in a hot air oven (Osworld). Stability chamber from ThermoLab India was used to perform stability studies.

### Selection of wavelength

100 µg/ml concentration of Beta-asarone primary stock solution has been prepared using methanol and subsequently diluted to obtain the secondary stock solution of 10 µg/ml. The samples were scanned using a UV-Vis Spectrophotometer within the range of 800-200 nm. The absorption maxima of Beta-asarone was found to be 306 nm. Therefore, this wavelength was selected as the detection wavelength for HPLC analysis.

### Optimized chromatographic conditions

The separation process was achieved using a Phenomenex C<sub>18</sub> column with dimensions of 250×4.6 mm, 5 µm. The mobile phase consisted of acetonitrile and a 10 mmol pH 4.0 ammonium acetate buffer in the ratio of 70:30% (v/v). The flow rate remained constant at 1.0 ml/min throughout the study. A volume of 20 µl was used for the injection and the column temperature was set at 25 °C. The detector (PDA) wavelength of 306 nm was selected and the run time was set to 10 min.

### Method validation

Validation was conducted in accordance with the ICH Q2R1 guideline for the parameters specified below [11].

### Specificity

The method's specificity was evaluated to confirm that there were no interferences from degradation products, excipients or other impurities in the active region. The HPLC system was injected with blank and Beta-asarone standard solution and the resulting chromatograms were recorded. The Shimadzu HPLC system, equipped with a Photo diode array detector was utilised to generate the peak purity of the marker Beta-asarone.

### System suitability

A 10 µg/ml concentration of standard solution containing Beta-asarone was introduced into the HPLC system. Parameters related to the peak, such as the area under the peak, the degree of tailing, the number of theoretical plates, retention time were observed and recorded.

### Linearity

A set of standard solution of Beta-asarone was prepared in the range of 0.2-3.0 µg/ml. Under ideal chromatographic conditions, the linearity was checked for the given concentrations and chromatograms were recorded for all the concentrations; each standard concentration was injected in triplicate (n=3). The linearity graph was established based on the average peak area and the marker concentrations.

### LOD and LOQ

Using slope and standard deviation, we computed the limit of Detection (LOD) and limit of Quantification (LOQ). Detection limit is generally determined by signal-to-noise ratios of 2:1 or 3 and quantitation limit is typically determined by signal-to-noise ratio of 10:1. To calculate LOD and LOQ the below formulae is used.

$$3.3 \times \frac{\sigma}{S} \quad 10 \times \frac{\sigma}{S}$$

### Accuracy

The method's accuracy was assessed through recovery studies, which involved the analysis of three distinct concentrations (80%, 100%, and 120% of the standard Beta-asarone solution) using high-performance liquid chromatography in three replicates.

### Precision

The study was performed by analysing the sample at Intra and Interday precision. The intraday precision test was performed on the same day, whereas the interday precision test was carried out on three different days. Under suitable chromatographic conditions, samples containing Beta-asarone were analyzed. The standard deviation (SD), mean and percentage RSD were calculated.

### Assay of Vacha churna formulation

A precise quantity of one gram of Vacha churna was weighed and subsequently added to a volumetric flask with a capacity of 10 ml. Then the flask was filled with methanol until it reached the specified mark. Subsequently the sample was subjected to the sonication for a period of 30 minutes. The required amount of solution is taken and centrifuged at a speed of 10,000 rpm for ten minutes. Then the supernatant liquid was collected for estimation by using RP-HPLC and the percentage recovery of marker is calculated [12].

### Stress studies

The stress studies were conducted according to the recommended conditions by ICH guidelines. The stress study of Beta-asarone was performed by subjecting it to various conditions like acidic, alkaline, oxidative, photolytic and thermal environments. The experiment was carried out in triplicates to observe the degradation profile [13-16].

### Acid hydrolysis

An acid degradation experiment was conducted by mixing 10 ml of the standard solution (200 µg/ml) of Beta-asarone with 40 ml of 0.1M Hydrochloric acid. The reaction was carried out by refluxing the mixture at 80 °C for 5 hours in a water bath. The sample under stress was cooled and neutralized using 0.1M Sodium hydroxide solution and subsequently diluted with the appropriate diluent. The sample then injected into the chromatographic system to observe the presence of any endogenous substances.

### Base hydrolysis

The experiment was carried out by combining 10 ml of the standard solution (200 µg/ml) of Beta-asarone with 40 ml of a 0.1M Sodium hydroxide solution. The reaction was executed by refluxing the above mixture by placing in a water bath at about 80 °C for 5 hours. After reflux, the stressed sample was cooled and neutralized by adding 0.1M Hydrochloric acid solution. Post-neutralization, the solution was then diluted with suitable diluent. The diluted solution was then loaded into the HPLC system.

### Oxidation

The hydrolysis of Beta-asarone was carried out at different concentrations of peroxide solution (H<sub>2</sub>O<sub>2</sub>). In the first part of the study, 3% (v/v) and 5% (v/v) H<sub>2</sub>O<sub>2</sub> was used for 24 h at room temperature but there is no sign of degradation. However, it was necessary to determine the possibility of the decomposition within 24 h, so the tests were conducted in 30% (v/v) hydrogen peroxide. Beta-asarone final solution (10 µg/ml) was analyzed using HPLC.

### Thermal degradation

Beta-asarone (10 mg) was subjected to high temperature through the hot air oven, which was heated up to 80 °C for 24 h. Afterwards, the marker was dissolved in methanol. Further dilution was conducted to reach a 10 µg/ml concentration for the Beta-asarone standard solution. The sample solution is then injected into the HPLC system to observe the presence of any degradants or impurities.

### Photolytic degradation

Approximately 10 mg of Beta-asarone was carefully measured and subjected to direct sunlight for a duration of 8 h. The marker

compound was subsequently diluted to a concentration of 10 µg/ml and then introduced into the chromatographic system.

#### Determination of shelf-life

The designed RP-HPLC method was employed to analyse the content of the marker. The stability study was performed following the guidelines outlined in the ICH Q1A(R2). The formulation was stored under accelerated conditions of temperature 40 °C±2 °C and relative humidity of 75%±5% RH for a period of 6 mo. The storage conditions for the long term required 30 °C±2 °C temperature and 65%±5% RH relative humidity for a period of 12 mo. The stability tests were conducted by placing the Vacha churna formulation in a stability chamber. The shelf life calculation of the formulation was performed by using the systat Sigma Plot software 15.0 version (www.systatsoftware.com), which has been approved by the USFDA [17, 18].

#### RESULTS AND DISCUSSION

According to literature review few analytical techniques such as HPTLC, HPLC, LC-MS and GC-MS were reported for identifying, separating and quantifying Beta-asarone from *Acorus calamus linn* [19-21]. *Sunitha Shailajan et al.*, has estimated and quantified Beta-asarone in the rhizome of *Acorus calamus*, as well as in various commercial formulations such as Sarasvata Churna, Maanasmithra Vatakam, Khadiradi Gutika, Chandraprabha Bati, Sanjeevani Vati, Mahashankh Bati, Smritisagar Ras, Abana, Vacadi Taila and Ashwagandharishtha [22]. *Avadhani et al.*, conducted an analysis using High Performance liquid Chromatography (HPLC) to estimate the content of Beta-asarone in 20 different species of *Acorus calamus*. The samples were collected from various regions in both South and North India [23]. *Shibin Felix et al.*, have gathered twenty distinct samples of *A. calamus* from various agroclimatic conditions in the Western Ghats region and analysed the amount of Beta-asarone present using RP-HPLC [24]. All previous techniques have concentrated on determining and measuring the amount of Beta-asarone present in the *A. Calamus* plant. Currently, there is a lack of stress studies, stability studies and shelf-life estimation for Vacha churna. A stability-indicating RP-HPLC method was developed and

validated for the current study and this method was also used for stability studies. The shelf life of Vacha churna was established through the analysis of Beta-asarone marker content, the selection of Beta-asarone for the RP-HPLC profile was based on its availability, feasibility and active concentration in the Vacha churna.

#### Development of the method

The UV spectrum of Beta-asarone was recorded in the range of 800 to 200 nm to determine the detection wavelength. Fig. 1 depicts a UV spectrum of the Beta-asarone. Several trials were conducted to determine the optimal buffer choice, pH of the mobile phase, mobile phase composition, flow rate and column oven temperature for the suggested RP-HPLC method. Finally, the ideal chromatographic parameters that were discussed in the method were successfully achieved. The Beta-asarone peak was detected at a retention time of 6.03 minutes, which is illustrated in fig. 2.

#### Method validation

##### System suitability

Six replicates of freshly prepared Beta-asarone were evaluated for system suitability test. The experimental parameters that were monitored for each injection are retention time, peak area, theoretical plates and tailing factor. The acceptance limits were met for the qualifying parameters, such as the %RSD for peak area and retention time was <2, tailing factor was <2 and theoretical plates were >2000. This demonstrates that the designed approach is apposite for the system. The table 1 displays the results of system suitability.

Table 1: Results of system suitability test

Parameter	Beta-asarone
% RSD of retention time of 6 injections	0.29965 %
% RSD of area of 6 injections	0.36966 %
Theoretical Plate count	13513
Tailing factor	1.202

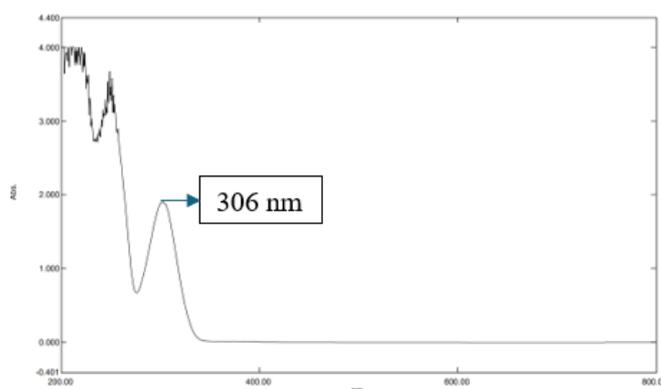


Fig. 1: UV spectrum of Beta-asarone

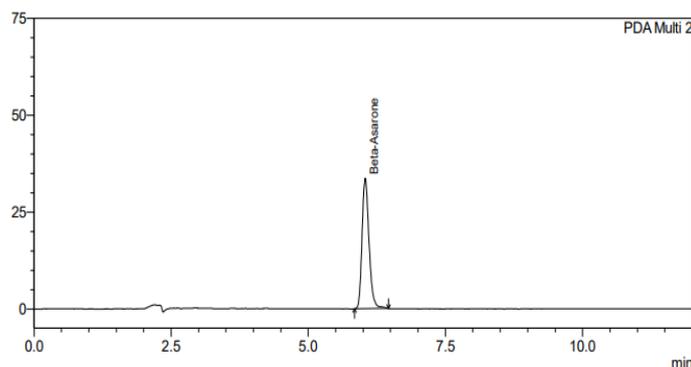


Fig. 2: Optimized chromatogram of Beta-asarone

### Specificity

The marker compound eluted at a retention time of 6.03 minutes. The specificity results indicates that there were no interference peaks at the retention time of the Beta-asarone. Hence, the lack of

interference peaks originating from degradants and impurities provides evidence that the proposed method is both selective and highly sensitive. The chromatograms of the Blank and Beta-asarone were represented as fig. 2 and 3. The fig. 4 represents the peak purity of the marker.

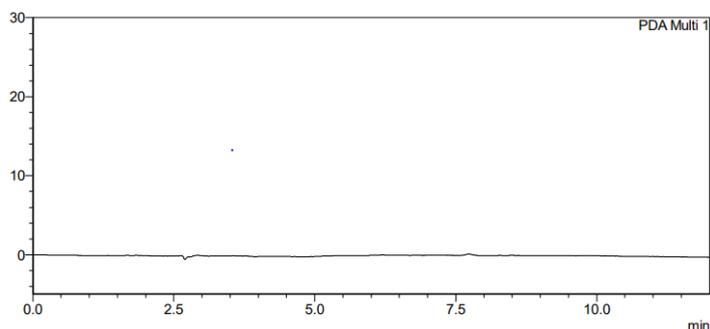


Fig. 3: Blank chromatogram

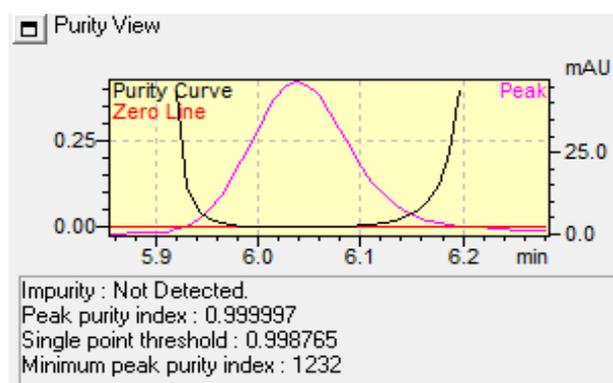


Fig. 4: Peak purity of beta-asarone

### Linearity and range

The relationship between analyte response and concentration variations is established through linearity, which is represented by a regression line derived from a mathematical equation. This relationship is often

quantified using the correlation value ( $R^2$ ) in the linear regression equation. In addition, the range is determined by the highest and lowest concentration values observed during the test. The study yielded an  $R^2$ -value of 0.9991 within the concentration range of 0.2-3.0  $\mu\text{g/ml}$ , as shown in table 2. The linearity plot was represented in fig. 5.

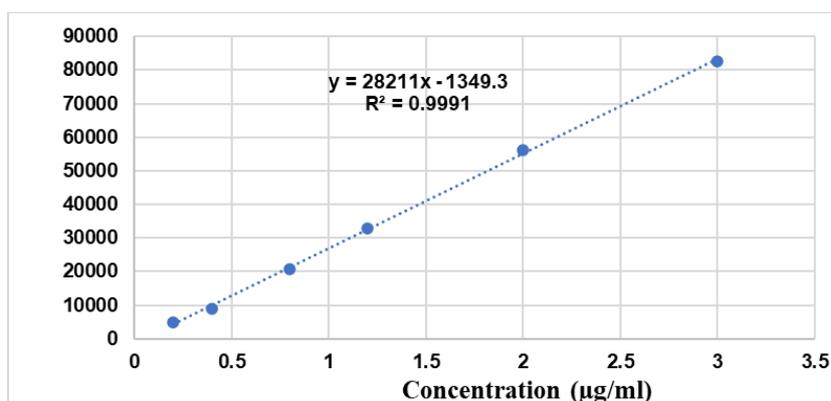


Fig. 5: linearity plot of beta-asarone

### Accuracy

Accuracy was determined at three distinct levels of concentration of 80%, 100% and 120% of the standard marker. The method

demonstrated a high level of accuracy, with an overall % recovery ranging from 99.06% to 103.76%. This suggests that this approach is appropriate for accurate quantification of Beta-asarone. Table 3 displays the overall results of validation.

**Precision**

A series of 6 injections of Beta-asarone was introduced into the HPLC system. Precision was evaluated for both interday and

intraday measurements. The mean, standard deviation and relative standard deviation (RSD) were calculated for peak area and retention time. The %RSD was determined to be within the specified limits.

**Table 2: Results of linearity data**

S. No.	Concentration ( $\mu\text{g/ml}$ )	Area average
1	0.2	4985
2	0.4	8824
3	0.8	20784
4	1.2	32864
5	2.0	56331
6	3.0	82517
Regression Equation		$y = 28211x - 1349.3$
Correlation Coefficient		$R^2 = 0.9991$
Slope		28211
y-Intercept		1349.3

**Table 3: Results of validation parameters**

Parameter	Beta-asarone	Acceptance criteria	Inference
Specificity	Marker peak is well resolved	Peak purity >0.999	Passed
LOD	0.00602 $\mu\text{g/ml}$	-	-
LOQ	0.0182 $\mu\text{g/ml}$	-	-
Accuracy 80%	99.06%	95.0% to 105.0%	Passed
100%	101.59%		
120%	103.76%		
Intraday precision	%RSD of peak area: 0.553008 %RSD of retention time: 0.24635	%RSD < 2 %	Passed
Interday precision	%RSD of peak area: 0.772607 %RSD of retention time: 0.49359		

**Assay of the formulation**

The %recovery of Beta-asarone in the formulation was estimated to be 100.45%. It demonstrates the appropriateness of the method for evaluating Beta-asarone in Vacha churna. The average recovery of Beta-asarone in the formulation is shown in table 4. Fig. 6 displays a chromatogram of Beta-asarone in Vacha churna.

**Stress studies**

According to the ICH recommendations stress study was performed to determine the degradation products in presence of various stress conditions. During the stress degradation studies, there is a chance that degradant peaks will be eluted alongside marker peak at the same retention time. Therefore, a photodiode array detector (PDA) is commonly employed to determine the peak purity index at the specific wavelengths of the marker. Based on the findings from the stress study, it is evident that the Beta-asarone withstands with acid hydrolysis and oxidation degradation conditions. Significant degradation was observed in the sample when exposed to alkaline

hydrolysis, sunlight and temperature. The obtained results confirm that the absence of any endogenous substances (impurities or degradants) under different stress conditions. The marker peak was not affected by any interference. The developed method is designed to be specific in determining Beta-asarone even in the presence of any degradant. The %degradation observed is shown in table 5.

**Table 4: % Recovery of Beta-asarone in Vacha churna formulation**

S. No.	% Recovery for Beta-asarone
1	100.24
2	100.73
3	100.38
Average	100.45
SD $\pm$	0.2523

All the values are presented as mean $\pm$ SD, n=3

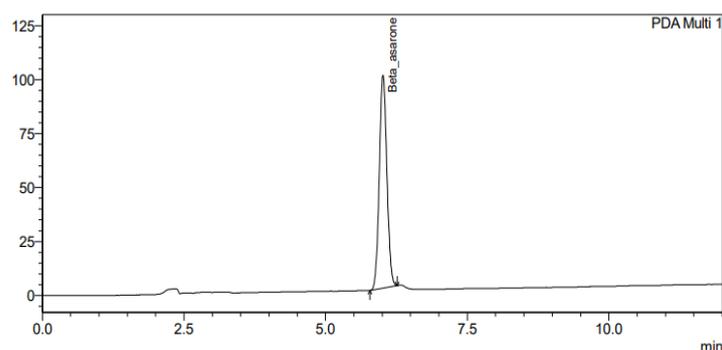
**Fig. 6: Chromatogram of Vacha churna**

Table 5: Results of stress study

S. No.	Type of degradation	% Degradation observed Beta-asarone	Peak purity
1.	Acid hydrolysis	16.41±0.72	Pass
2.	Alkali hydrolysis	23.95±0.13	Pass
3.	Oxidative degradation	19.62±0.28	Pass
4.	Thermal degradation	21.83±0.64	Pass
5.	Photolytic degradation	25.27±0.18	Pass

All the values are presented as mean ±SD, n=3

### Shelf-life determination

A stability study was conducted to determine the shelf life of classical Ayurvedic formulation vacha churna by analysing the Beta-asarone content under accelerated and long-term storage conditions. The marker concentration in the Vacha churna was analysed by using high-performance liquid chromatography under

accelerated conditions at time intervals of 1, 3 and 6 mo and 3, 6, 9 and 12 mo at long-term storage condition. The shelf life calculation was performed using Systat sigma plot software. The investigated shelf life for Vacha churna using Beta-asarone was determined to be 62.40 mo, as depicted in fig. 7. The chromatograms obtained from the stability studies were showed in fig. 8-15. Table 6 represents the average marker content over time.

Table 6: Average content of Beta-asarone in the formulation

Storage condition	Time (Mo)	Average marker content of Beta-asarone (%)
Initial timepoint (T <sub>0</sub> )	0	100.12±0.12
Accelerated condition	1	99.62±0.16
	3	99.18±0.21
	6	98.76±0.19
Long-term condition	3	99.95±0.24
	6	99.43±0.17
	9	99.06±0.26
	12	98.72±0.22

All the values are presented as mean±SD, n=3

Shelf Life Analysis

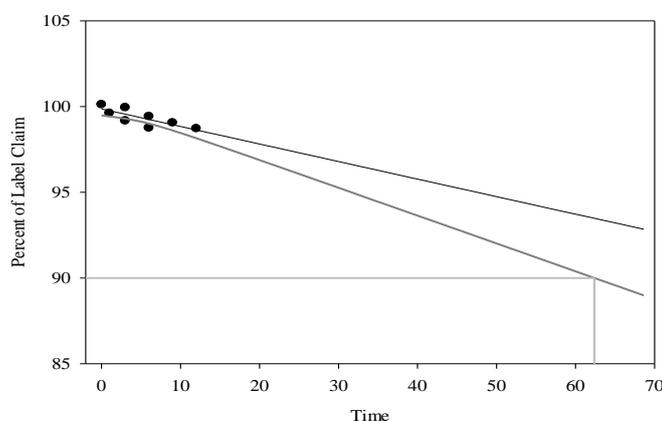
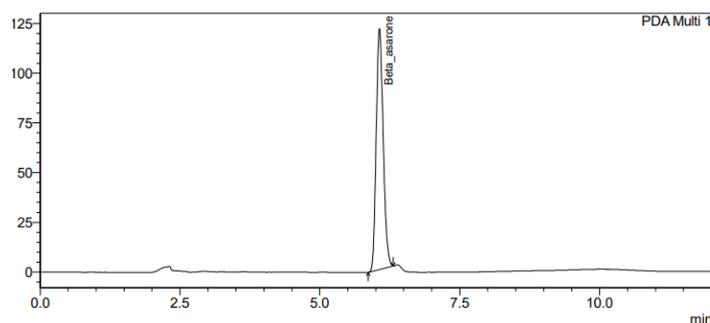
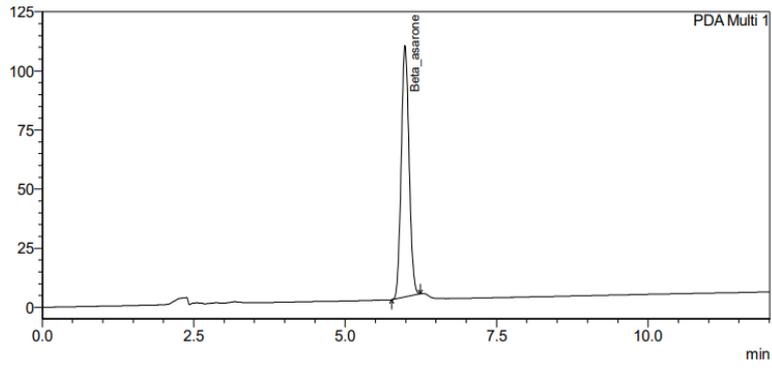
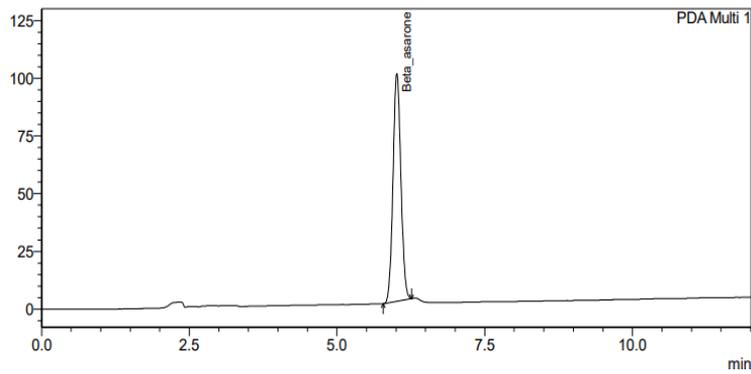


Fig. 7: Shelf-life of Vacha churna (62.40mo) considering the marker content analysis of Beta-asarone

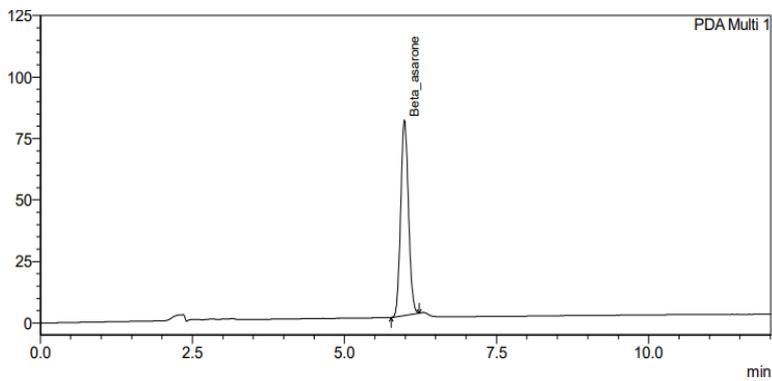
Fig. 8: Initial time point (T<sub>0</sub>) chromatogram of Vacha churna



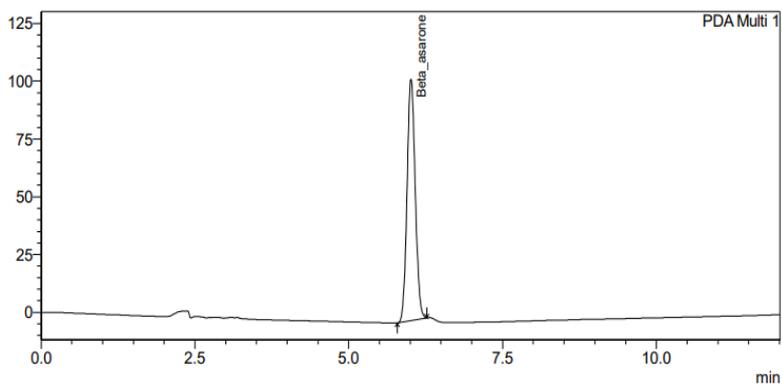
**Fig. 9: Accelerated 1<sup>st</sup> mo time point chromatogram of Vacha churna**



**Fig. 10: Accelerated 3<sup>rd</sup> mo time point chromatogram of Vachachurna**



**Fig. 11: Accelerated 6<sup>th</sup> mo time point chromatogram of Vachachurna**



**Fig. 12: Long term 3<sup>rd</sup> mo time point chromatogram of Vacha churna**

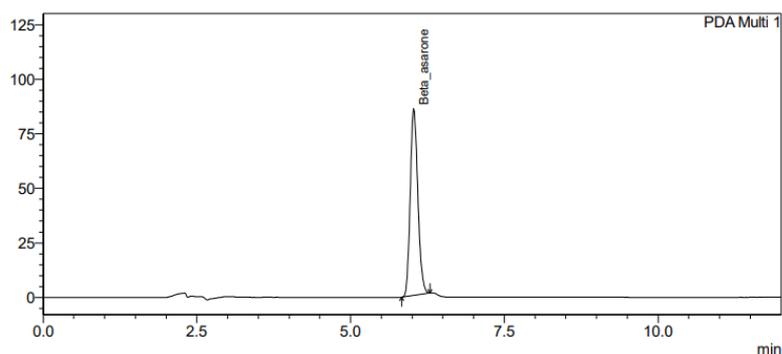


Fig. 13: Long term 6<sup>th</sup> mo time point chromatogram of Vacha churna

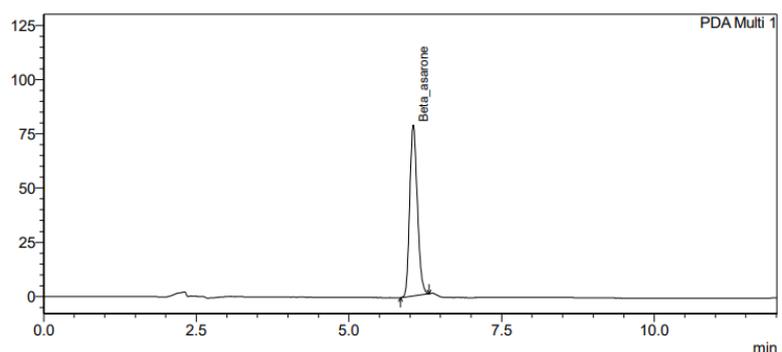


Fig. 14: Long term 9<sup>th</sup> mo time point chromatogram of Vacha churna

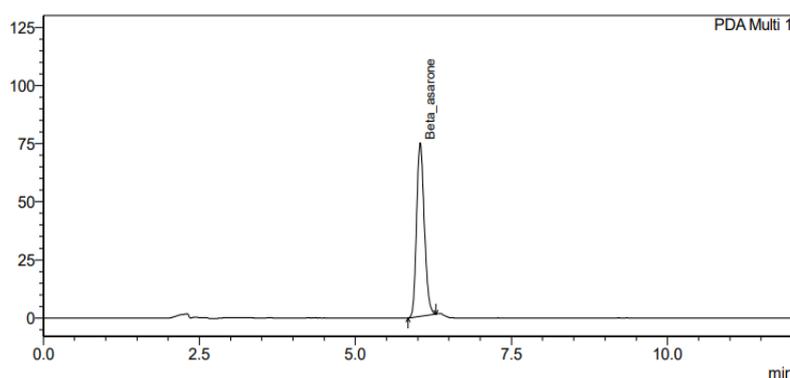


Fig. 15: Long term 12<sup>th</sup> mo time point chromatogram of Vacha churna

## CONCLUSION

Ensuring the quality of Ayurvedic formulations during storage and transportation is essential for maintaining stability. Therefore, accurately estimating the shelf life is crucial for maintaining quality. Nevertheless, the intricate composition of these phytoconstituents causes a significant obstacle in establishing specific range and standards for quality. An order was issued by the Ayush ministry on August 12<sup>th</sup>, 2016, which states that the Ayurvedic medicines to indicate their shelf life based on the research findings. The date of expiry of the medicine must be determined through stability studies conducted in accordance with the guidelines outlined in the Ayurvedic Pharmacopoeia of India. It is necessary to evaluate the shelf life of these traditional formulations with empirical evidence. The investigated shelf life of Vacha churna which was determined by using Beta-asarone is found to be 62.40 mo. Based on the analysis, it has been found that the investigated shelf life of the formulation is greater than the labelled shelf life of 24 mo for churna formulation as per the gazette notification. Further real time studies can provide substantial evidence on the commercial batches.

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Gundawar Ravi, Resources: Niharika Gaddam, S. G. Vasantharaju Muralidhar Ballal. Data curation: Niharika Gaddam, Gundawar Ravi and Basavaraj S Hadapad. Writing original draft, preparation: Niharika Gaddam, B. S. Muddukrishna and Krishnamurthy Bhat. Writing, review and editing: Niharika Gaddam, S. G. Vasantharaju and Syeda Alisha Md Isha Ali. All authors have read and agreed to the published version of the manuscript.

#### CONFLICT OF INTERESTS

All authors declare that they do not have any conflicts of interest.

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