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Development and Validation of Stability Indicating HPTLC Method for Estimation of Embelin in *Embelia tsjeriam* cottam

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ABSTRACT

Embelin, a naturally occurring benzoquinone, is obtained from fruits of *Embelia tsjeriam cottam,* which plays a vital role in the Ayurvedic system of medicine. An approach for the stress degradation was successfully applied for the development of stability indicating HPTLC method for the determination of Embelin. It was spotted on the plates precoated with silica gel 60 F_{254} and developed using toluene: ethyl acetate: formic acid, (6:3.5:0.5 v/v/v) as mobile phase. Densitometry analysis was carried out at 291 nm. The method showed high sensitivity with good linearity over the concentration range of 200-1000ng/spot with correlation coefficient value 0.997. The accuracy of the method was established based on the recovery studies. The LOD and LOQ were 6.09ng/band and 18.48ng/band respectively. The system showed a peak for Embelin at R_f of 0.68 ± 0.02.The aim of our study was to observe the effect of various stress conditions on this potential drug candidate. Stress testing of Embelin was carried out according to the International Conference of Harmonization (ICH) guideline Q1A (R2). Embelin was subjected to stress conditions of acid, alkaline hydrolysis, oxidation, photolysis and thermal degradation. A significant degradation was found to occur by acid and oxidation hydrolysis and to a lesser extent under thermal stress and alkaline hydrolysis; and much lesser with photolytic stress. Stress degradation studies on embelin provide an insight into its stability.

Keywords: *Embelia tsjeriam cottam,* Embelin, HPTLC, Validation, stress degradation.

INTRODUCTION

Embelin (2, 5-dihydroxy-3-undecyl-p-benzoquinone), is found to be the active principle of *Embelia tsjeriam cottam* commonly known as vidanga. Vindanga is official in Indian pharmacopeia 2014 [1] and Ayurvedic Pharmacopia. [2] *Embelia tsjeriam cottam* belonging to the family Myrsinaceae, is a climber found in the Western Ghats of Lonavala and also seen in the southern states of Maharashtra, Karnataka, Kerala, Tamil Nadu and Andhra Pradesh up to an altitude of 1600 m. Fresh fruits are eaten as raw for rheumatoid inflammation. The chemical constituents of this plant are embelin a benzoquinone [3], fatty ingredients: christembine-alkaloids, resinoids, tannins and volatile oils. [4] Embelin shows diverse pharmacological activities including chemo prevention in hepato-carcinogenesis observed in Wistar rats [5], anti-fertility effects [6], wound healing [7], antibacterial [4, 8], free radical scavenging [9] and *in vitro* cytotoxic activity. [10] The fruits of this plant contain (2.5-3.1%) embelin on dry weight basis. *Embelia tsjeriam cottam* finds its use in several Ayurvedic preparations like *Sanjivani Vati, Pippalyasavam, Dhanwantara ghritham*, *Vidangarishta*,

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Kaisoraguggulu vatica. [11] Quality control for herbal preparations or products however is much more difficult than for synthetic drugs because of chemical ingredients complexity and any loss in particular chemical may result in loss of pharmacological action of that herb. [12] Literature survey reveals UV spectrophotometric method reported for the estimation of Embelin in plant and in pharmaceutical dosage form [13-14], HPLC [15], RP-HPLC [16] and HPTLC [17], HPLC stress degradation studies. [18] To the best of our knowledge, no stability indicating HPTLC method has been reported for Embelin. In this study, the effect of different stress conditions on the stability of Embelin was observed using High-Performance Thin layer Chromatography (HPTLC) analysis as per ICH guidelines.

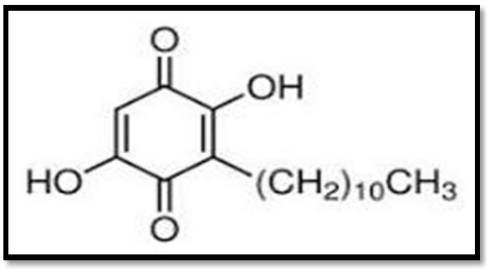

Fig. 1: Structure of Embelin

MATERIAL AND METHODS

Chemical and Reagents: Vidanga seeds were purchased from a local market in Pune. Embelin was purchased from Yucca enterprises, Mumbai and was used as such, without any further purification. Aluminum sheets precoated with silica gel (60 F_{254}, 20 cm × 20 cm with 250µm layer thickness) were purchased from E-Merck, Darmstadt, Merck (Germany). Methanol (HPLC grade), Toulene (AR grade), Ethyl acetate (AR grade), Formic acid (AR grade) were purchased from S. D. fine chemical Laboratories, Mumbai. Hydrochloric acid (HCl), hydrogen peroxide (H_2O_2, 30% v/v) and sodium hydroxide (NaOH) were purchased from LOBA CHEMIE PVT. LTD., Mumbai.

Equipment: Chromatographic separation of drug was performed on aluminum plates precoated with silica gel 60 F_{254}, (10 cm × 10 cm with 250µm layer thickness). Samples were applied on the plate as a band with 6mm width using Camag 100µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland).

Optimized Chromatographic conditions: The mobile phase was composed of Toulene: Ethyl acetate: Formic acid (6:3.5:0.5 v/v). 10 cm × 10 cm CAMAG twin trough glass chamber was used for linear ascending development of TLC plate under 20 min saturation conditions and 10 ml of mobile phase was used per run, migration distance was 90 mm. Densitometric scanning was performed using Camag TLC scanner 3, operated by win CATS software (Version 1.4.3, Camag), slit dimensions were 5.00 × 0.45 mm and Deuterium lamp was used as a radiation source.

Preparation of Standard stock solution: Standard stock solution was prepared by dissolving 10 mg of Embelin in 10 ml of methanol to get concentration of 1000µg/ml. From the standard stock solution, working standard solution was prepared containing 100µg/ml of Embelin.

Preparation of sample solution (Formulation Analysis):

Formulation analysis is carried out as per label claim of the marketed formulation (Vidanga Ghana) which is claimed to contain 250 mg of aqueous extract of vidanga. The assay was carried out by weighing tablet equivalent to 0.025 g of extract in 10 ml of methanol from which 5ml of solution was diluted to get a extract solution. Analysis was repeated two times. 5 and 10µL of sample solution were applied on 10 × 10 cm pre-coated TLC plate as a band length 4 mm for the assay of Embelin. The content of Embelin was calculated upon extrapolation from standard marker in linearity study:

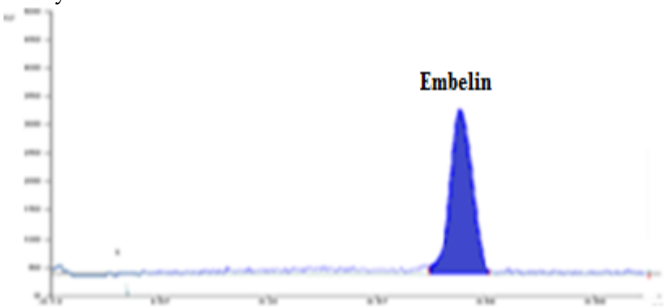

Fig. 2: Densitogram of Embelin

Stress Degradation Study

Stress degradation studies were carried under condition of acid/ base/ neutral hydrolysis, oxidation, dry heat and photolysis. For each study, samples were prepared as per ICH Q1A(R2) [19-20]

1. The blank subjected to stress in the same manner as the drug solution
2. Embelin working standard solution subjected to stress condition.

Dry heat and photolytic degradation were carried out in solid state. 10µl of the resultant solution was then applied on TLC plate and densitogram was developed.

Stress conditions were optimized in terms of strength of reagent and time of exposure to obtain 10-30% degradation.

Alkaline hydrolysis

1 ml working standard solution of Embelin (1000µg/ml) was mixed with 1 ml of 0.1 N NaOH (Methanolic) and volume was made up to 10 ml with methanol. The solution was kept for 24 hours in dark place. 4µl of the resulting solution was spotted on TLC plate. Average 82.44% of Embelin was recovered with no peak of degradation in alkaline condition.

Acidic hydrolysis

1 ml working standard solution of Embelin (1000µg/ml) was mixed with 1 ml of 0.1 N HCl

(Methanolic) and volume was made up to 10 ml with methanol. Solution was kept for 24 hours in dark place. 4µl of the resulting solution was spotted on TLC plate and densitogram was developed. Average 74.68% Embelin was recovered with one peak of degradation product (D1) at the R_f value of 0.24 (Figure 3 I & II).

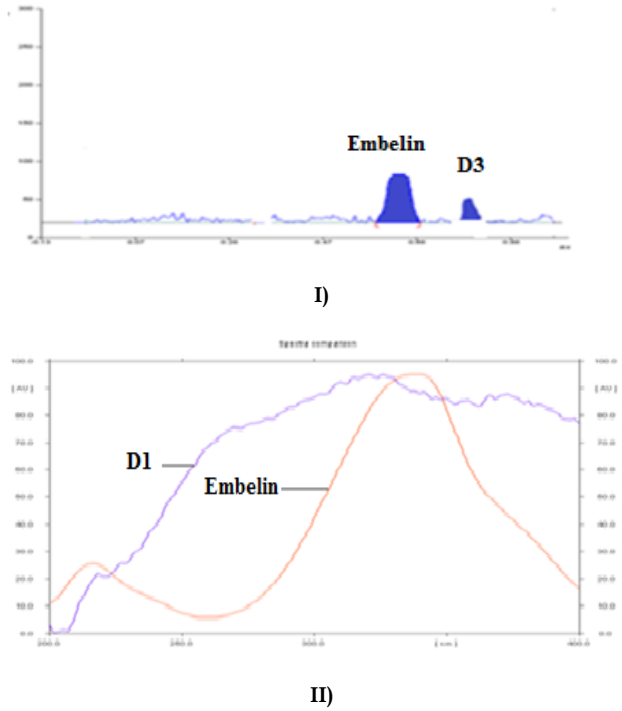

Fig. 3: I) Densitogram of acid treated Embelin, II) UV overlay spectrum of Standard Embelin, acid treated Embelin, Degradation product (D1).

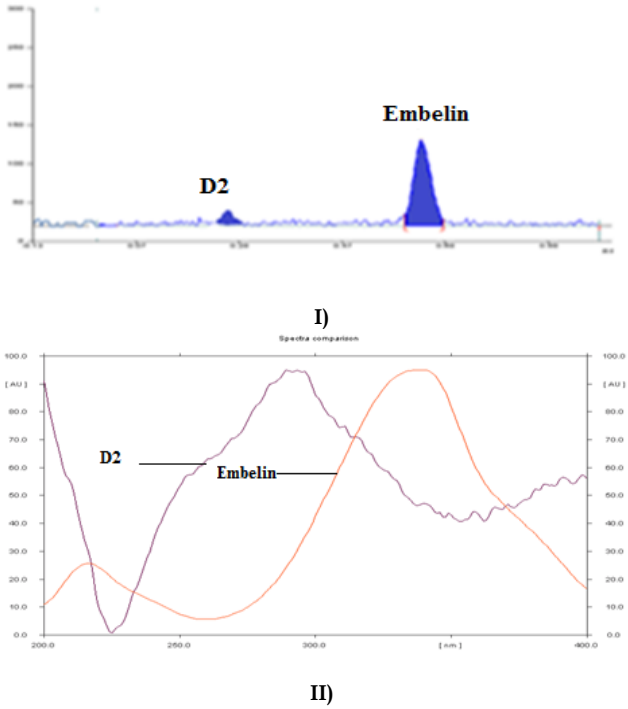

Fig. 4: I) Densitogram of H_2O_2 treated Embelin, II) UV overlay spectrum of Standard Embelin, H_2O_2 treated Embelin, Degradation product (D2).

Neutral Hydrolysis

1 ml working standard solution of Embelin (1000µg/ml) was mixed with 1 ml water and volume made up to 10 ml with methanol. The solution was kept for 24 hours in dark place. 4µl of the resulting solution was spotted on TLC plate. Average 85.40% of Embelin was recovered with no peak of degradation in neutral condition.

Oxidation

1 ml working standard solution of Embelin (1000µg/ml) was mixed with 1 ml of 3 % v/v solution of H_2O_2 and volume made up to 10 ml with methanol. The Solution was kept for 6 hours in dark place. 4µl of the resulting solution was spotted on TLC plate and densitogram was developed. Average 79.18% Embelin was recovered with one peak of degradation product (D1) at the R_f value of 0.20 (Figure 4 I & II).

Degradation under dry heat

Dry heat studies were performed by keeping drug sample in oven (60^0 C) for a period of 8 hour. A sample was withdrawn after 8 hour. 4µl of the resulting solution was spotted on TLC plate and densitogram was developed. Average 78.57% Embelin was recovered with one peak of degradation product (D1) at the Rf value of 0.24 (Figure 5 I & II).

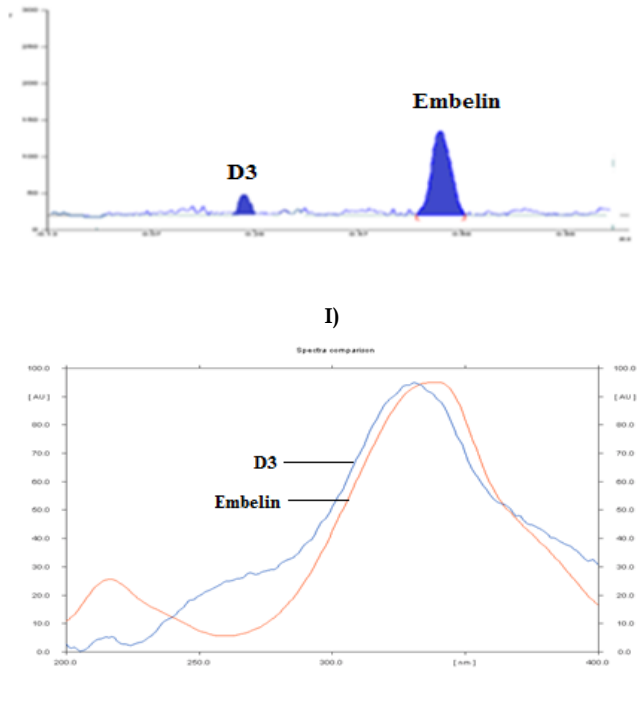

Fig. 5: I) Densitogram of Embelin exposed to dry heat, II) UV overlay spectrum of Standard Embelin, dry heat treated Embelin, Degradation product (D3).

Photo-degradation studies

Photolytic studies were carried out as per ICH guidelines [21] by exposure of Embelin to UV light up to 200 watt hours/square meter and subsequently to cool fluorescent light to achieve an illumination of 1.2 million Lux Hrs. Sample was weighed, dissolved in methanol and appropriate dilutions were made to get final concentration of 100 µg/ml. 4µl of the resulting solution was spotted on TLC plate. Average 81.27% of Embelin was recovered with no peak of degradation.

Table 1: Summary of stress degradation of Embelin

Stress Degradation Condition	Peak Area	% Recovery	Rf of degradation product	Peak purity	
				R (s, m)	R (m, e)
Base (0.1 N NaOH, kept for 24 hrs)	8568	82.44	-	0.9994	0.9998
Acid (0.1 N HCl, kept for 24 hrs)	7924	74.68	0.79	0.9996	0.9998
Neutral (kept for 24hr)	8801	85.40	-	0.9997	0.9996
H_2O_2 3% (kept for 6 hrs)	8311	79.18	0.19	0.9994	0.9991
Heat dry (60°C, 8 hrs)	8263	78.57	0.21	0.9994	0.9998
Photo stability (UV, 200 watt hrs/square meter and Florescence 1.2 million Lux. Hrs)	8476	81.27	-	0.9998	0.9994

The results of stress degradation studies of Embelin obtained by us fairly match the ones reported by Ferreira G and Laddha KS. [18]

Method Development and Validation of HPTLC Method

The proposed analytical method was validated according to ICH guidelines [19] with respect to parameters such as Specificity, linearity, Precision, Accuracy, Sensitivity and Robustness

Specificity: The specificity was carried out to determine whether there are any interference of any impurities (presence of components may be expected to be present) at retention factor of Embelin.

Linearity: It is the ability to attain test results that are directly proportional to the concentration of analyte in the sample. The linearity of the method was established by a spotting a series of sample of Embelin, the solutions of five different concentration levels 200-1000ng/band was applied to the TLC plate. Calibration curves for the standard Embelin were constructed by plotting the peak area against their respective concentrations, linear regression was applied and slope, intercept, and correlation coefficient- R^2 were determined.

Precision: Express the closeness of agreement between the series of measurement obtained from multiple sampling of same homogeneous sample under the prescribed conditions. System precision was determined in terms of repeatability.

Intraday precision: Precision of the system was evaluated by analyzing six independent standard solutions of 200ng/band for Embelin was applied on TLC and the peak area was determined and expressed as a mean and %RSD calculated from the data obtained.

Interday precision: Precision of the system was evaluated by analyzing three independent standard preparations on three different days and %RSD calculated from the data obtained.

Accuracy: Accuracy is determined in terms of percentage recovery. The accuracy study was performed at 80%, 100% and 120% for Embelin. Standard and sample solutions were applied to TLC plate in triplicate and percentage recoveries of Embelin were calculated. The area of every level was used for calculation of % recovery.

Sensitivity: The Limit of Detection (LOD) is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The Limit of Quantitation (LOQ) is the minimum injected amount that produces quantitative measurements with acceptable precision in chromatography.

The limit of detection (LOD) and Limit of Quantitation (LOQ) were determined using following formulae;

$$LOD = 3.3(SD)/S$$

$$LOQ = 10\ (SD)/S$$

Where, SD = Standard Deviation of lowest response

S = avg. of the slope of the calibration curve

Robustness: Robustness of the developed method was investigated by evaluating the influence of small deliberate variations in procedure. Variables like change in mobile phase composition (± 2.0%), change in chamber saturation time, change in time from spotting to development and development to scanning and the effects on the R_f values and area were noted.

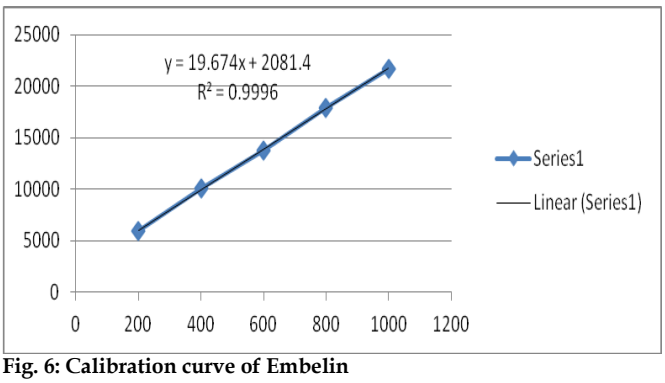

Fig. 6: Calibration curve of Embelin

RESULT AND DISSCUSSION

Stress degradation of Embelin was carried out as per ICH guidelines. The results indicate that Embelin is prone to oxidative, thermal and acid catalysed hydrolytic degradation. A well resolved product of degradation was obtained under these conditions. This method can distinguish between standard and degraded Embelin. The optimised HPTLC method was validated as per ICH Q2R1 guidelines. The results are as follows

Specificity: The specificity of the method was ascertained by peak purity profiling studies. The peak purity value was found to be more than 0.997, indicating the non interference of any other peak of degradation product or impurity.

Linearity: In the linearity parameter calibration curve was constructed by plotting peak area against respective concentration of Embelin. The plots were found to be linear in the range of 200-1000ng/band with coefficient of correlation (r2) 0.999 for Embelin as shown in Table 2 below.

Table 2: Linearity of Embelin

S. No.	Conc (ng/band)	Peak Area
1	200	5931
2	400	10927
3	600	13756
4	800	17893
5	1000	21722

Accuracy: The accuracy study was performed by standard addition at 80%, 100% and 120% level to pre-analysed marketed sample. The percentage recovery was found to be 98.49, 98.72, 101.67% presented in Table 3 below.

Table 3: Recovery studies of Embelin

Level %	Sample	Standard	Conc (ng/band)	Avg Area	% Recovery
80	400	320	720	15579	95.30
100	400	400	800	17578	99.68
120	400	480	880	18154	92.85

Precision: The inter-day and intra-day precision of the system was ascertained from determinations of peak areas of sample solution. The % Relative Standard Deviation for system precision is presented in Table 4 and 5 below.

Table 4: Inter-day precision study of Embelin

Conc (ng/band)	Inter-day mean area	SD	% RSD
200	5624	50.08	1.90
400	10378	56.63	1.04
600	14354	65.96	0.57

Table 5: Intra-day precision study of Embelin

Replicate	Intraday
1	5704
2	5680
3	5677
4	5666
5	5659
6	5648
SD	17.22
%RSD	0.644

Table 6: Robustness Study

S. No.	Parameter	Robust condition	% RSD
1	Saturation time (20 min) ± 2 min	18min	0.125
		22min	0.129
2	Mobile phase composition- Toluene: Ethyl acetate: Formic acid (6:3.5:0.5) ± 0.2 ml	Toluene: Ethyl acetate: formic acid (6.2:3.5:0.5)	0.110
		Toluene: Ethyl acetate: formic acid (6:3.2:0.5)	0.215
3	Time from spotting to development (immediate)	After 30 min	0.212
		After 2 hours	0.315
4	Time from development to scanning (immediate)	After 2 hours	0.276
		After 24 hours	1.214

Sensitivity: Limit of Detection (LOD) and LOQ of Embelin was found to be 6.09ng/ band and 18.48ng/band respectively. The LOD and LOQ showed that the method is sensitive for Embelin.

Robustness: Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase ratio, Variables like change in mobile phase composition (± 2.0%), change in chamber saturation time, change in time from spotting to development and development to scanning and the effects on the R_f values and area were noted as shown in Table 6.

Assay: The assay study was performed for vidanga Ghana tablet and standard. The results are tabulated in Table 7 below

Formulation details:

Formulation Name	Manufacturer	Mfg. Date	Expiry Date
Vidanga Ghana	Chaitanya Pharmaceuticals, Pvt., ltd	September 12	September 15

Table 7: Assay for Embelin from Vidanga Ghana tablet

Volume applied (µl)	Area	% Assay
5	6523	1.62
10	7129	1.79

The developed method was found to have all parameters within limits of ICH guidelines. Since the stress degradation products of Embelin are well resolved, this method has stability indicating property. It may be used in phytopharmaceuticals formulation industry to monitor the stability of active marker compound, to analyse the plants for Embelin content before taking up the production. It can serve as a Quality control parameter for herbal raw materials containing Embelin.

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