

ORIGINAL RESEARCH ARTICLE

Quantitative and Chromatographic Fingerprint Analysis of *Embelia ribes churna* Formulations by HPLC Method

Sudani R.J*, Akbari B.V¹, G. Vidyasagar², P.Sharma³

*Singhania University, Pachari Bari, Jhunjhunu, Rajasthan, India

¹Shree Dhanvantary Pharmacy College, Kim, Surat, Gujarat, India

²Dean, Faculty of Pharmaceutical Sciences, Kachchh University, Bhuj, Kutch, Gujarat, India

³Vidyasthali Institute of Technology, Science and Management, Jaipur, Rajasthan, India

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ABSTRACT

Embelia ribes burmf., also known as Vidanga, is one of the oldest herbs in Indian traditional medicine. It is used mainly as an anthelmintic, carminative and stimulants. A selective, precise and accurate High Performance Liquid Chromatography (HPLC) method has been developed for the quantification of Embelin in Vidanga churna formulations. The chromatographic separation was performed on Chromatopak Peerless basic C₁₈ column (250 mm L x 4.6 mm ID Column; packing size- 5 μ) with a mobile phase Methanol: Phosphate Buffer pH 3.0 (adjusted with 5% v/v acetic acid) in 90:10 proportion at flow rate was 1.4 ml/min. Densitometric analysis was carried out in the absorbance mode at 291 nm. Developed HPLC method showed good regression ($r^2 = 0.9988 \pm 0.0012$) and the recovery of Embelin was in the range of 99.6 – 102.2%. The limit of detection and limit of quantitation were found to be 1.94 μ g/ml and 5.891 μ g/ml respectively. The method was validated for precision, recovery, limit of detection and limit of quantitation as per ICH guidelines. The proposed HPLC method was found to be simple, precised and accurate and can be used for the quantitative and chemical fingerprint analysis of Vidanga.

KEY WORDS: Vidanga, Embelin, HPLC, Fingerprint analysis

INTRODUCTION

In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects. In olden times, vaidyas used to treat patients on individual basis, and prepare drug according to the requirement of the patient. But the scene has changed now; herbal medicines are being manufactured on a large scale in pharmaceutical units^[1]. Medicinal plants have played a key role in world health. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants^[2-4].

The construction of chromatographic fingerprints plays an important role in the quality control of complex herbal medicines^[5]. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the “chemical integrities” of the herbal medicines and therefore be used for authentication and identification of the herbal products. Based on the concept of phytoequivalence, the chromatographic fingerprints of herbal medicines could be utilized for addressing the problem of quality control of herbal medicines^[6]. By definition, a chromatographic fingerprint of herbal medicine is, in practice, a chromatographic pattern of pharmacologically active and or chemically characteristic constituents present in the extract^[7, 8]. Thus many kinds of chemical fingerprint

analysis methods to control the quality of herbal drugs have gradually come into being, such as thin layer chromatography, gas chromatography, high performance liquid chromatography etc.

HPLC is a popular method for the analysis of herbal medicines because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. In general, HPLC can be used to analyze almost all the compounds in the herbal medicines. Reversed- phase (RP) columns may be the most popular columns used in the analytical separation of herbal medicines. It is necessary to notice that the optimal separation condition for the HPLC involves many factors, such as the different compositions of the mobile phases, their pH adjustment, pump pressures etc. Thus, a good experimental design for the optimal separation seems in general necessary [9-11]. In order to obtain better separation, some new techniques have been recently developed in research field of liquid chromatography. These are micellar electro kinetic capillary chromatography (MECC) [12], high-speed counter-current chromatography (HSCCC), low- pressure size-exclusion chromatography (SEC) [13], reversed-phase ion-pairing HPLC (RP-IPC-HPLC) [14, 15] and strong anion exchange HPLC [16]. They will provide new opportunities for good separation for some specific extracts of some herbal medicines.

Embelia ribes burm f. also known as Vidanga [17], is one of the oldest herbs in Indian traditional medicine. Embelia ribes have a long history of use in ayurvedic system of medicine in various forms like churna, asava, aristha, lauha and taila. It is an Indo-Malaysian species, mainly found in India, Sri Lanka, Singapore, and Malaysia. In India it is majority found in central and lower Himalayas, Arunachal Pradesh, Assam, Bengal, Orissa, Andhra Pradesh and Madhya Pradesh [18]. It is available throughout India up to an altitude of 5000 feet [19]. The main active component is Embelin, chemically 2, 5-dihydroxy-3-undecyl-1,4-benzoquinone [Fig.1]. Embelin is occur in golden yellow needles and is insoluble in water but soluble in alcohol, chloroform and benzene. Other components are christembine, qercitol, vilangin and resinoid [20]. It is mainly used as an anthelmintic, carminative and stimulant. It is also used in treatment of abdominal disorders, lung diseases, constipation, indigestion, fungus infections, mouth ulcer, sore throat, pneumonia and obesity [21].

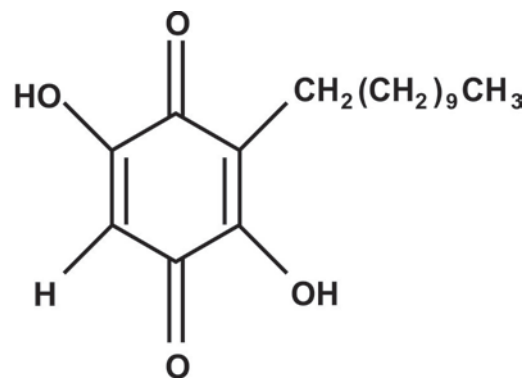


Figure 1: Structure of Embelin

In the present study, HPLC method have been developed as chemical fingerprints for Vidanga churna for three different formulations using Embelin as an active chemical marker. The developed method were validated in terms of accuracy, specificity, precision, linearity range, robustness, limit of detection (LOD) and limit of quantitation (LOQ) as per ICH guidelines. The developed method can also be used as a quality control tool.

MATERIAL AND METHOD

Dried ripe fruits of *Embelia ribes* Burm. f., family- Myrsinaceae were procured from local market of Guwahati, Assam in the month of November. The collected fruits of *Embelia ribes* were authenticated at Botanical survey of India, Pune. All the chemicals used were of HPLC grade obtained from Merck chemicals, India. HPLC analysis was performed on a Perkin Elmer Series 200 HPLC system using Photo diode array and UV visible detector. The chromatographic separation was performed on Chromatopak Peerless basic C₁₈ column (250 mm x 4.6 mm ID Column; packing size- 5 μ). The mobile phase Methanol: Phosphate Buffer pH 3.0 (adjusted with 5% v/v acetic acid) in 90:10 proportion were used with a flow rate of 1.4 ml/min. The injection volume was 20 μ l. The detection wavelength was set at 291 nm. The chromatographic conditions are summarized in (Table 1).

Table 1: chromatographic conditions

Parameters	Optimized condition
Column	Chromatopak (ODS) Peerless basic C ₁₈ column (250 mm L x 4.6 mm ID Column)
Mobile phase	Methanol: Phosphate Buffer pH 3.0 (90:10 v/v)
Flow rate	1.4 ml/min
UV detection	291 nm
Injection	20 μ l
Temperature	Ambient

Preparation of extracts of marketed formulations

Marketed formulations (F-I, F-II and F-III) were macerated with chloroform at room temperature for 48 hrs in dark. The extract was filtered and concentrated over water bath till a dry residue was obtained. This extract was further used for HPLC analysis.

Preparation of standard solution and calibration curve

10 mg of Embelin dissolved in sufficient volume of methanol and sonicated for 2 min. Then volume was made up to 10 ml with methanol. Appropriate aliquots from above stock solutions were taken in different 10 ml volumetric flasks and diluted with methanol up to the mark to obtain final concentrations 10-60 µg/ml. These solutions were filtered through 0.22µ filter paper and were subjected to HPLC analysis. Calibration curves were constructed by plotting average peak area versus concentrations and regression equations was computed for Embelin.

Preparation of sample solution

Dried chloroform extract equivalent to 10 mg of Embelin of F-I, F-II, and F-III respectively was dissolved in sufficient volume of methanol and sonicated for 10 min. After sonication the solution was filtered through Whatman filter paper no.41 and then volume was made up to 10 ml with methanol. Appropriate aliquot from the stock solution was transferred to a 10 ml volumetric flask and the volume was made up to the mark with mobile phase to obtain a solution containing 30 µg/ml of Embelin. This solution was filtered through 0.22 µ filter paper. The sample solutions were prepared in triplicate and 20 µl volume of each sample solution was injected twice into the sample injector of RP-HPLC under the optimized chromatographic conditions. The concentrations of the drugs in samples were calculated by measuring their peak areas and comparing with peak areas of pure drug solutions of respective concentrations.

Preparation of Mobile Phase

Solution of phosphate buffer was prepared by dissolving 3.01 gm of Potassium dihydrogen phosphate and 5.04 gm of di-sodium hydrogen phosphate in 1000 ml of HPLC grade water and its pH was adjusted to 3.0 by 5 % v/v glacial acetic acid. Mobile phase was prepared by mixing methanol with solution of phosphate buffer pH 3.0 in the ratio of 90:10 v/v, filtered through 0.22 µm membrane filter paper and

then sonicated using ultrasonicator for 15 min^[22].

Validation of method

The developed method has been validated in terms of linearity, precision, specificity, robustness and accuracy as per ICH guidelines^[23]

Linearity and range

The stock solution 1000 µg/ml of Embelin was prepared in methanol and this stock solution was diluted with methanol to obtain final concentrations of 10-60 µg/ml of Embelin for HPLC analysis. A 20 µl volume of each sample solutions were injected into LC six times, under the optimized chromatographic conditions.

Limit of detection and Limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined using following formulae.

$$\text{LOD} = 3.3(\text{SD})/\text{S}$$

$$\text{LOQ} = 10 (\text{SD})/\text{S}$$

Where, SD = Standard Deviation of response

S = avg. of the slope of the calibration curve

Precision

Precision of the method was verified by repeatability (intra-day) and intermediate (inter-day) precision studies. Intra-day and inter-day precision were performed by analyzing sample solution of 30 µg/ml of embelin six times on the same day and three different days respectively. Measurement of peak area for active compound was expressed in terms of % Relative Standard Deviation (% RSD)

Accuracy

Accuracy of the method was tested by carrying out recovery studies at different spiked level by standard addition method. Standard Embelin solution was added at three different levels (80,100 and 120%). At each level three determinations were performed and results were calculated by the difference between the spiked and un-spiked sample analyzed under the same conditions.

Robustness

To determine the robustness of the developed method, few parameters such as variation in composition of mobile phase, flow rate and wavelength were deliberately varied. Each factor was changed at three levels and one factor was changed at one time to estimate the effect.

Quantitative analysis of embelin in marketed formulations

For assay of marketed formulations F-I, F-II and F-III, extracts were prepared as mentioned in the above section and subject to optimized HPLC conditions.

RESULTS AND DISCUSSION

Method Development and Optimization

Initially many solvent systems were tried. Different system parameters such as composition of mobile phase, method of sample preparation and flow rate were modified to obtain well resolved chromatogram with maximum number of plates. Initially different combinations of mobile phases with different concentration of phosphate buffer with methanol and acetonitrile were tried in order to determine

the best conditions for the Embelin. The mobile phase consisting of methanol: phosphate buffer pH 3 adjusted with 5 % v/v glacial acetic acid (90: 10 v/v) was optimized as it was found to give best system suitability test parameters.

HPLC fingerprint analysis of marketed formulations

As (Figure 2) clearly indicate characteristic peak of embelin showed characteristic peak of embelin at retention time 5.6 ± 0.01 min. To obtain the fingerprints of three marketed formulations (F-I, F-II and F-III), chromatogram of all three formulations (Figure 3, 4 and 5) were overlaid with the chromatogram of isolated chemical marker embelin as shown in (Figure 6) which clearly indicates common peak in all the formulations at same retention time as that of embelin.

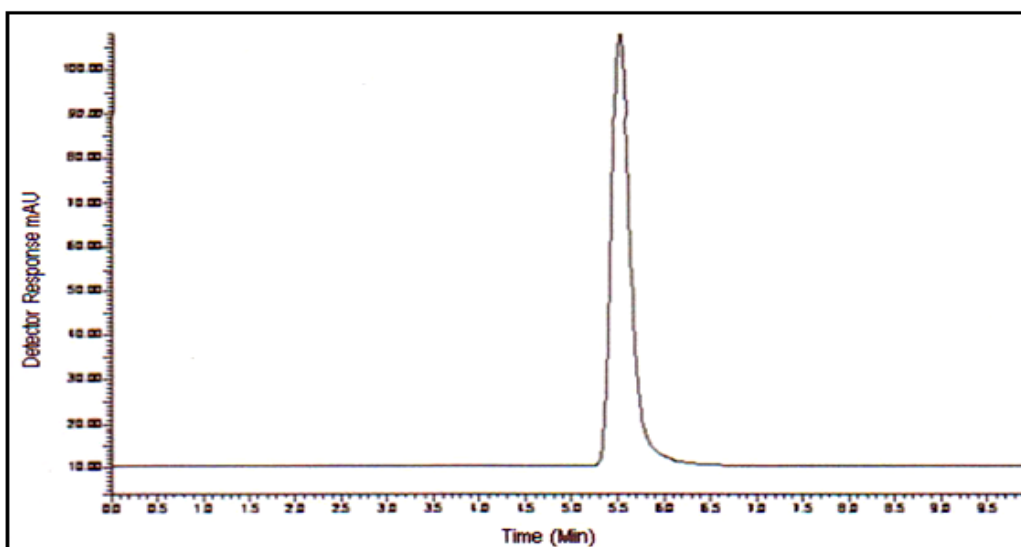


Figure 2: HPLC chromatogram of embelin [Methanol: Phosphate buffer (90:10 v/v) pH 3 adjusted with acetic acid]

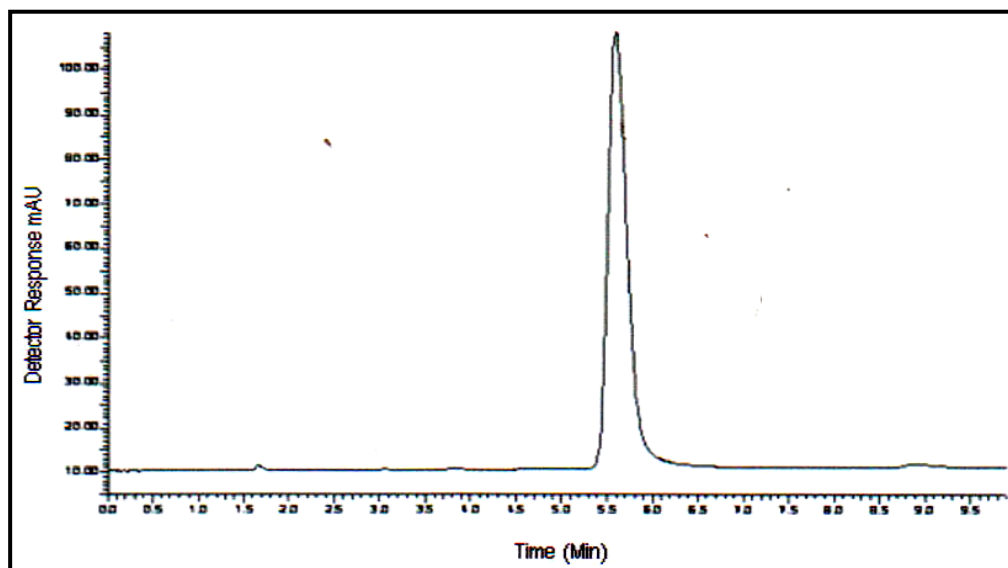


Figure 3: HPLC Fingerprint of F-I

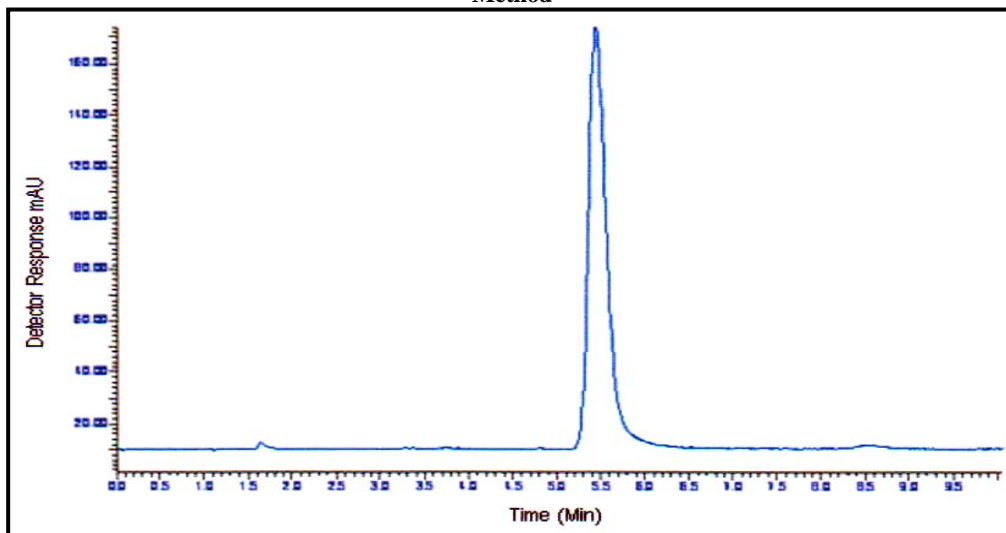


Figure 4: HPLC Fingerprint of F-II

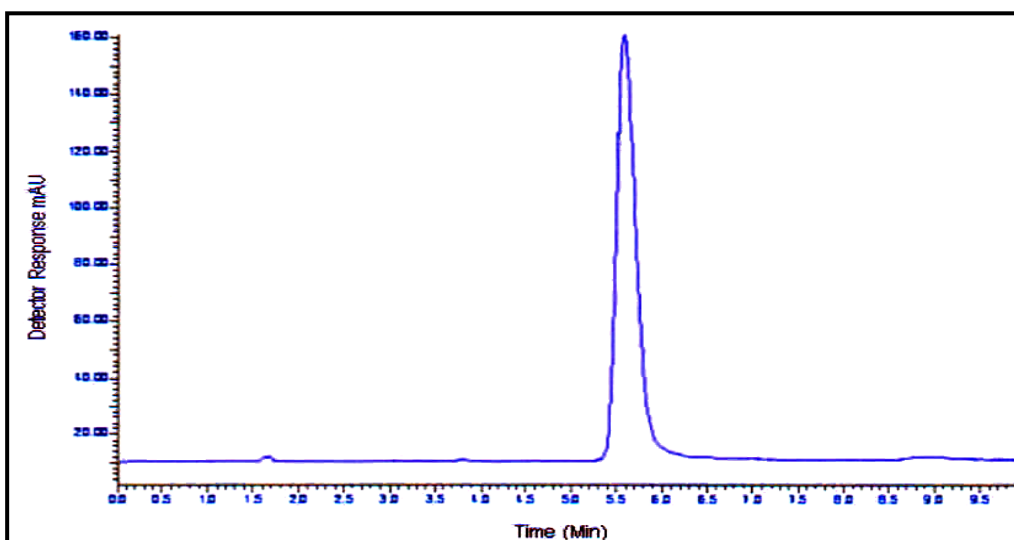


Figure 5: HPLC Fingerprint of F-III

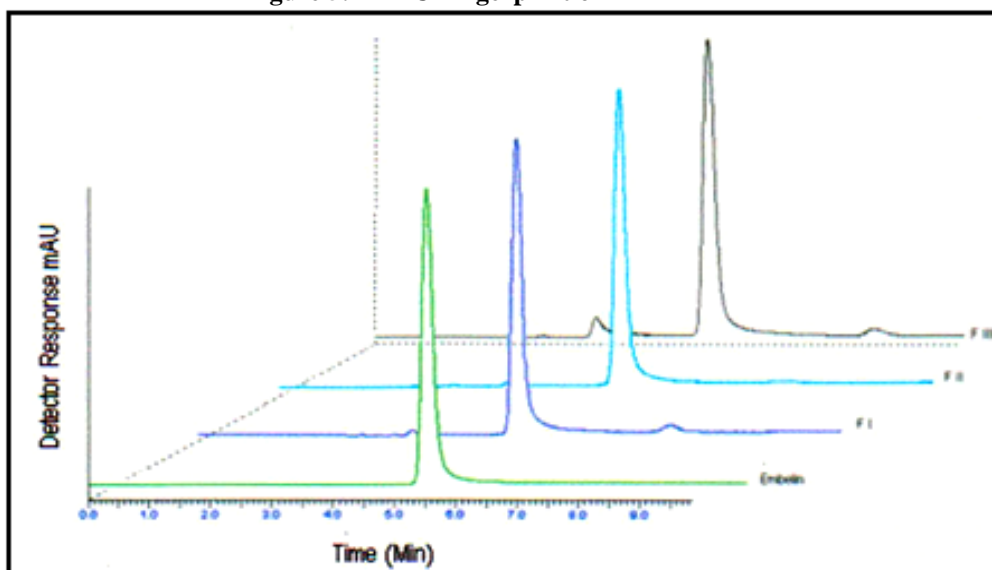


Figure 6: 3D overlain chromatogram of embelin and marketed formulations

Validation of method

Linearity plots and detection limits

The linearity for embelin was established by plotting the peak area versus concentration. The

linearity of calibration curves was verified by correlation study and the correlation coefficients were found to be 0.9989. The LOD and LOQ for embelin were found to be 1.94µg/ml and 5.891µg/ml respectively as shown in (Table 2).

Table 2: Calibration curves, Limit of detection and Limit of quantitation of Embelin

Linear range*	Correlation coefficient	LOD*	LOQ*
10-60 µg/ml	0.9989	1.94 µg/ml	5.891 µg/ml

* Results are mean of six determinations

Precision, accuracy and robustness

As shown in (Table 3), % RSD value for inter-day and intra-day precision based on peak measurement was found to less than 2 (n=6), hence the method were found to be precise. The

average recoveries of the embelin were in the range of 99.61-102.2%. Satisfactory recoveries with small % relative standard deviations (less than 2) were obtained, which indicate the accuracy of the method. The robustness study revealed that there were no significant differences in peak areas and retention time after small, deliberate variation of the analytical conditions, which evaluated the robustness of the proposed method.

Table 3: Precision and Accuracy for Embelin

Formulation	Precision (%RSD)		Recovery*					
			80%		100%		120%	
	Interday	Intraday	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
F-I	0.45	0.35	100.26%	1.2	100.6%	0.52	99.79%	0.187
F-II	1.4	0.72	100.2%	1.4	102.2%	1.11	99.72%	0.67
F-III	1.52	1.33	100.08%	0.28	99.61%	0.78	99.94%	1.17

* Results are mean of three determinations

Quantitative analysis of embelin in marketed formulations

A single peak at retention time of 5.6 min was observed in the chromatogram of the embelin extracted from formulations. There was no interference from the other components present in formulations. The concentration of embelin in formulations was calculated by measuring their peak areas and comparing their peak areas of standard drug solution. Results are shown in (Table 4).

Table 4: Quantitative analysis of Embelin from Marketed formulations

Formulation	Concentration	% Purity*	Embelin content* (w/w)
F-I	30 µg/ml	96.12%	2.08%
F-II	30 µg/ml	99.97%	2.19%
F-III	30 µg/ml	100.4%	2.21%

* Results are mean of three determinations

CONCLUSION

The proposed HPLC method was developed and validated for determination of Embelin. The method was found to be simple, sensitive, accurate, rugged, robust, rapid and precise. Hence, the above said method could provide an important reference to establish the quality control method for other Vidanga medicinal preparations for manufacturers as well as different regulatory agencies.

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