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RESEARCH ARTICLE

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Development and Validation of a Liquid Chromatography Method for Determination of Embelin in Crude Extract of *Embelia ribes*

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Abstract
This study was designed to develop and validate a liquid chromatographic method for determination of Embelin in crude extract of *Embelia ribes*. The chromatographic separation was performed on a HPLC coupled to a diode array detector with Thermo Hypersil-Keystone, BDS C-18 column (25 cm L x 4.6 mm ID; particle size- 5µ) using methanol and 0.1% TFA (88:12 v/v) as mobile phase, at a flow rate of 1 ml/min. Quantification was achieved with UV detection at 288nm, based on peak area. The optimized procedure involved the extraction of Embelin in crude extract of *Embelia ribes* by soxhlet and maceration using chloroform and methanol as a solvent. Extraction by soxhlet at 70°C for 2 hr using chloroform as solvent was the best suited method. The assay method was developed and validated for system suitability, linearity, range, accuracy, precision and robustness. The plot of integrated peak area and concentration of Embelin was found to be linear over a range of 6.25-200 µg/ml. The LOD and LOQ (µg/ml) levels were, 1.5 and 4.5, respectively. The recovery experiments led to mean recovery of 98.6%. This indicates that this method is simple, rapid, sensitive, precise and accurate and can be applicable for the extraction and quantitative determination of Embelin in crude extracts of *Embelia ribes*.

Keywords: Extract of *Embelia ribes*, Embelin, HPLC.

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INTRODUCTION

Herbal medicines have gained great popularity in developing countries owing to their natural abundance and lesser side effects. *E. ribes*, popularly known as 'Vidanga' or 'Vavding' in Ayurveda, is an important medicinal plant belonging to family *Myrsinaceae*. It is also known as 'false black pepper'. It has restricted and sporadic distribution mainly in the Western Ghats and Eastern Himalayas of India [1]. It is also a critically endangered species of conservation importance in India [2, 3].The plant is a scandent shrub, and is used in a large number of Ayurvedic formulations.

E.ribes (Embelia fruits) have cardioprotective effect against isoproterenol –induced myocardial infarction in albino rats as reported by Bhandari et al, 2008 [4]. Fruits are also reported as diuretic, carminative, anti-bacterial, anti-inflammatory and anti-astringent in literature [5]. Also fruit decoction is useful in fevers and diseases of chest and skin. Decoction of the leaves is used as a blood purifier [6]. Antidyslipidemic effect of E.ribes in diabetic rats has also been reported by Bhandari et al, 2002 [7]. *E. ribes* is also used as a medicine for influenza [8].

E.ribes yields Embelin (embolic acid / 2, 5-dihydroxy-3-undecyl-2, 5-cyclohexadiene-1, 4-benzoquinone) (Figure 1) and other highly valued secondary metabolites, which have a wide range of clinical applications. Embelin, the main phyto-constituent of *E. ribes*, is used as anthelmintic, and for control of conception [9, 5]. It is also used in bronchitis, mental disorders, jaundice, skin diseases and leprosy [6,10].

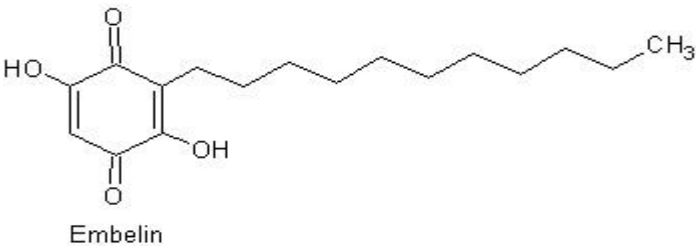

Figure 1. Structure of Embelin

The impediments in the acceptance of the herbal drugs are the lack of standard quality control profiles. The quality of herbal medicine, that is, the profile of the constituents in the final product has implication in efficacy and safety. Due to the complex nature and inherent variability of the chemical constituents of the plant based drugs, it is difficult it establish quality control parameters. The modern analytical techniques such as HPTLC, HPLC etc are accepted to help in circumventing this problem [11].

Sudani et al, 2011, have reported the quantitative and chromatographic fingerprint analysis of *Embelia ribes* churna formulations by HPLC method [12]. Embelin has also been quantified in hexane extract, chloroform extract; methanol and water extract using RP-HPLC method by Shelar et al, 2009 [13]. For routine analysis of Embelin using same proportion of mobile phase attempts have been made to develop, optimize and validate the HPLC method for quantitative estimation of Embelin in the crude extract (methanol and chloroform) of dried E.ribes fruits, using soxhlet and maceration extraction techniques, which make it different from the studies reported earlier. This method was fully validated and successfully used for routine analysis of Embelin content in the crude extract of *E. ribes*.

METHODOLOGY

Chemicals and Reagents

Authentic reference standard of Embelin (≥98% pure) was procured from Sigma Aldrich (St. Louis, U.S.A.). HPLC grade methanol, tri-fluoro acetic acid and chloroform were procured from Merck Specialty Private Ltd (Mumbai, India), BDH and Qualigens Fine Chemicals Pvt Ltd. (Mumbai, India) respectively.

Instrumentation

The liquid chromatographic system consisted of Agilent 1200 Infinity series containing 1260 Quat Pump VL, with UV/visible detector 1260 DAD VL. Chromatographic analysis was performed using Thermo Hypersil-Keystone, BDS C-18 column with 25cm x 4.6mm internal diameter and 5µm particle size. Data acquisition was performed using EZChrom Elite software, (Agilent Technologies). Mettler Toledo (Model: XP-205) and Mettler (Model: AE 160) balances were used for weighing purpose. Rotary vacuum evaporator of Prama Solutions (Model: IKA RV10) was used for concentrating the extract.

Experimental Design

Optimization of Extraction Conditions

Extraction conditions were optimized with two different extraction solvents (chloroform and methanol) using two different extraction processes (soxhlet and maceration). Briefly 50 g of *E. ribes* dried fruit grounded to powder was extracted with 250 ml solvent (chloroform and methanol separately) by soxhlet at 70°C for 2 hour and maceration for 48 hours at room temperature under dark conditions. The filtered extract was concentrated under reduced pressure to 5 ml. The resulting concentrate was appropriately diluted with methanol and injected into the HPLC to assess the best suited conditions. The yield obtained was higher with chloroform as an extraction solvent using soxhlet extraction process at 70°C.

Preparation of Standard Stock Solution

A standard stock solution was prepared by accurately weighing 5mg of Embelin and transferring it to a 5ml-volumetric flask. Methanol was added to dissolve and volume was made up to 5ml to obtain a solution of

1mg/ml of Embelin. This solution was stored at refrigerated conditions and used for further dilutions.

Preparation of Sample.

0.5ml of concentrated crude extract in chloroform was taken in a separate 100ml volumetric flask and made upto the volume with methanol. 5 ml of this solution was taken in a 10 ml volumetric flask and volume was made up to the mark with methanol. The sample solution was allowed to stand for 5 min and filtered. 10 µl of this solution was injected to carry out the assay. Embelin content in E.ribes extracts was calculated based on calibration curve.

HPLC Conditions

RP-HPLC assays were carried out on a Thermo Hypersil-Keystone, BDS C-18 column with 250 x 4.6mm internal diameter and 5µm particle size, Part No 28105-060. The mobile phase consisted of Methanol (A) and 0.1 % TFA (B) (in proportion of 88:12 v/v). It was degassed before use. The analysis was carried out at 288nm and column temperature was set at 30°C. Before injection, samples were filtered through 0.45µm disposable filters. The chromatographic conditions are mentioned in Table 1.

Mobile Phase	**Methanol : 0.1% TFA (88:12 v/v)**
Stationary Phase	C-18 column with 25cm x 4.6mm internal diameter and 5µm particle size
Wavelength	288nm
Flow Rate	1ml/min
Injection Volume	10µl
Temperature	30°C
Mode of Operation	Isocratic

Table 1: Chromatographic Condition for *E. ribes* extract

Method Validation

The developed HPLC method was validated according to International Conference on Harmonization (ICH) guidelines [14]. The method was validated for linearity, range, accuracy, precision, LOD, LOQ and robustness. Precision of the method was carried out by system precision using six injections of standard and by method precision using six independent test injections. LOD was calculated as 3.3 times the standard deviation and LOQ as 10 times the standard deviation. The accuracy was evaluated using three injections at three different concentrations and recovery studies were done by using established calibration curve. Robustness was done by changing flow rate, wavelength and column temperature, one at a time with three injections each. During, this period the sample and standard solutions were kept at 2-8°C.

System suitability

System suitability test was done to verify the suitability of the instrument. A system suitability solution of 0.2 mg/ml of Embelin standard was prepared and 10 µl of the standard was injected six times to check the performance of the instrument. The parameters established were percentage relative standard deviation (%RSD) for peak area, tailing factor and theoretical plates.

Results and Discussion

Optimization of Extraction Method

This research has focused on the quantification of Embelin from crude extract of *E. ribes* fruits. The chromatographic condition of the proposed HPLC method was studied and optimized with respect to the extraction solvent. Table 2 shows the amount of Embelin obtained with two different methods of extraction, soxhlet and maceration and with two different solvents. The highest yield of Embelin was obtained by soxhlet extraction with chloroform solvent. The described chromatographic conditions gave a clear baseline and sharp peak without peak tailing of Embelin (Figure 3), similar to that of Embelin standard (Figure 2). Quantification of the method was achieved with UV detection at 288nm based on the peak area. The average retention time was 7.2 min. The system suitability test results are given in Table 3.

S.No.	Replicate	Extraction technique	Extraction solvent	Embelin (mg/ml)
1	Replicate 1	Soxhlet	Chloroform	0.4063
2	Replicate 2			0.4083
3	Replicate 1	Soxhlet	Methanol	0.0258
4	Replicate 2			0.0258
5	Replicate 1	Maceration	Chloroform	0.0162
6	Replicate 2			0.0165
7	Replicate 1	Maceration	Methanol	0.0005
8	Replicate 2			0.0005

Table 2 : Yield of Embelin with Soxhlet and Maceration using the solvents Chloroform and Methanol.

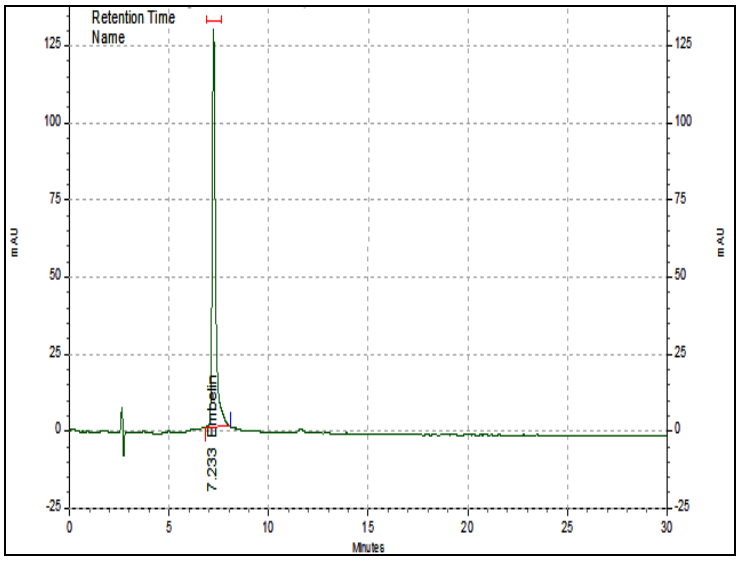

Figure 2 : Chromatogram of Embelin standard.

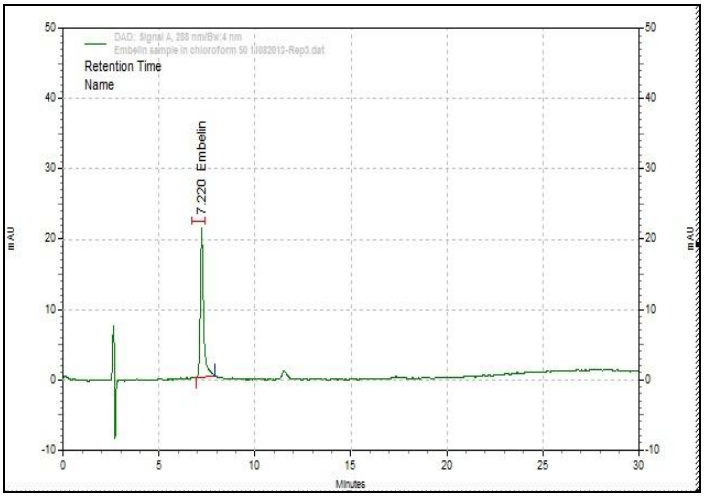

Figure 3 : Chromatogram of chloroform extract of *Embelia ribes.*

S.No.	Retention time, min	Peak Area of Embelin standard (mAU)	No. of Theoretical plates	Tailing Factor
1	7.2	16419714	10748	1.86
2	7.2	16476863	10546	1.81
3	7.2	16283521	10663	1.82
4	7.2	16091493	10682	1.81
5	7.2	16014974	10618	1.88
6	7.2	16272756	10519	1.86
Average	7.2	16259887	10629.3	1.84
%RSD	0	1.11	0.81	1.65

Table 3 : The system suitability test results of the developed method for determination of Embelin in *E. ribes*.

Method Validation

Linearity

The linearity of HPLC method was assessed by injecting six different concentrations of standard solution in range of 6.25µg/ml-200µg/ml. Each concentration was injected three times to provide information on variation in peak area values between replicates of same concentration. Linearity was assessed by plotting a calibration curve of Embelin concentration v/s peak area, and calculating the slope, y-intercept and coefficient of variation (R^2). The curve was found to be linear in the range of 6.25-200µg/ml. R^2 was observed to be 0.998. The linear equation was: y=75765x-19980, where y= peak area of Embelin and x= concentration of standard.

Range

The calibration curve was established using practical range, necessary to give accurate, precise, and linear results. It was determined by injecting standard solution at ten different concentrations with three replicates each and the average of each replicate was considered to find out the range. It was found out to be from 1.562-800 µg/ml.

Accuracy and precision

Accuracy was determined by spiking known amount of standard at three different levels (95%, 105% and 115% of the final concentration) with sample. The recovery studies were performed at three concentrations and each concentration was injected in triplicate. Accuracy of the method was calculated from % recovery and the results were found to be satisfactory. The percentage recoveries calculated were 96.01%, 100.35% and 99.72 respectively after spiking (Table 4).

S. No.	Replicate	% Conc. Spiked	Spiked peak area	% Recovery	Mean Recovery (%)
1.	Replicate 1	95%	9777541	96.01	98.69
	Replicate 2		9769782		
	Replicate 3		9709434		
2.	Replicate 1	105%	13303903	100.35	
	Replicate 2		13266989		
	Replicate 3		13030033		
3.	Replicate 1	115%	15157320	99.72	
	Replicate 2		15908468		
	Replicate 3		14800196		

Table 4 : Accuracy data of Embelin

Precision was determined by system precision and method precision. System precision was carried out by running six replicate injections from the same vial of Embelin standard (200µg/ml concentration). Method precision was established by determining the assay in six different preparations of a homogenous sample of *E. ribes*. The %RSD for system precision and method precision were 1.10% and 1.77% respectively.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ levels were determined by using the formula based on the standard deviation of the response and the slope. LOD and LOQ were calculated by using equations,

$$LOD = 3.3 * \sigma/slope$$

$$LOQ = 10 * \sigma/slope,$$

Where, σ = Standard Deviation,

Slope = Slope of the calibration curve

LOD was found to be 1.51µg/ml and LOQ was found to be 4.57µg/ml

ROBUSTNESS

Robustness of the method was studied by varying the flow rate (±10%), wavelength (±2 nm) and HPLC column temperature variation (±5°C) using a column heater temperature deliberately with three replicates

each. Only one parameter was changed at one time to estimate the effect. The method was found to be robust over a range of flow rate (0.9-1.1ml/min), wavelength (286-290nm) and column temperature (25°-35°C).

CONCLUSION

The method developed, optimized and validated is simple, accurate, linear, precise, and robust under the conditions of the experiment. This method is isocratic with uncomplicated mobile phase, and with simple sample extraction and preparation step. The resolved peak obtained was free from interferants indicating specificity of the method. Also, the method assures prolong life of the column and system due to lower percentage of acid in the mobile phase. Therefore, we suggest this method for routine analysis of Embelin in crude extracts of *E. ribes* in herbal quality control laboratories.

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