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REVERSE PHASE HPLC METHOD FOR QUANTITATIVE DETERMINATION OF EMBELIN IN POLY HERBAL FORMULATION

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ABSTRACT

Embelin is a unique chemical compound found in nature, composed of quinone moiety resembling Coq10 (Ubiquinones), having ketone and hydroxy groups. A Optimum solvent for extracting embelin from Embelia ribes and its simultaneous determination of content of embelin in the extract and content of embelin in Formulation which is available in market has been done in Gradient HPLC with less runtime of 10 minutes. Satisfactorily the results were obtained with respect to LOQ (0.5ppm), LOD (0.17 ppm) and Linearity (Correlation: 0.9976), Ruggedness (RSD: 0.8%), Accuracy (86%), Precision (RSD: 1.0%). The Present method was sensitive, accurate, simple reproducible and therefore can be recommended for using in Quality control to check the content of Embelin.

KEYWORDS: **Embelin; Quantitative analysis; Formulation; RP-HPLC; Validation.**

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INTRODUCTION

Herbal drugs are in great demand in the developed world for primary health care because of their efficacy, safety and lesser side effects. They also offer therapeutics for age related disorders like memory loss, osteoporosis immune disorders .In India despite its rich traditional knowledge heritage of herbal medicines and large biodiversity has a monotonous share of the world market due to export of crude extracts and drugs. The World Health Organization (WHO) recently defined traditional medicine including herbal drugs as comprising therapeutic that have been in existence, often for hundreds of years, before the development of modern medicine and are still in use today. The earliest recorded evidence of their use in Indian, Chinese Egyptian, Greek, Roman and Syrian texts dates back to about 5000 Years [1] . Embelia ribes is most widely used in tradition herbal medicine in India. Vidanga is a well known home medicinal product used as a home remedy and in Sanskrit it is named as "Jantanasa" [2]. The name suggests its fine action as Germicidal and Fungicidal The fruits Mainly Contains Benzoquinione derivatives Such as Embelin (2,5-dihyroxy-3-undecyl- 2,5 Cyclohexadiene-1,4- enzoquinone) and Vilangin[3]. The dried fruit has been used as an alternate Medicine for Antihelmentic, it also possess antiferlity [5], astrigent, carminative stimulant, antioxidant[4], anti-permetogenic, antibacterial[6], anti-inflammatory, antitumour[9], and anticancer activity[7]. Its activity against cancer therapy is being widely studied, it prevents the development of chemical carcinogen induced heptacarcinogenesis in rats [8,9] . The chemical structure of Embelin slightly resembling the structure of naturally occurring co-enzyme Q10 (Ubiquinones) , and it places an important role in Various Biological protective mechanism. The principle constituents are Embelin, quercizol, fatty ingredients and alkaloid, Christemine, a resinoid, tannins and minute quantitites of volatile oil . Embelin occur in golden yellow needles insoluble in water and soluble in alcohol, chloroform and benzene. Although many researchers have successfully isolated and extracted, there are few analytical methods reported in literature. Therefore, the aims of this study were to develop and validated HPLC with less runtime. The Present work also involves to find out the content of embelin in different solvent extracts [10,11,12] and formulation sample available in market.

MATERIALS AND METHODOLOGY

Instrument

HPLC experiments were performed using a waters alliance system equipped with a degasser, quaternary solvent mixer, auto sampler and a waters 996 Diode array detector. Initial development has been performed in PDA, to ensure the purity of the peak to ensure that there is no co-elution with principle peak. The experiments performed in 200-400nm range and extracted at 280 nm, whereas in this wavelength Embelin shows maximum absorbance. Initial development started with Pecosphere c18 (33*4.6 mm*3.0µ) column for achieving runtime 10 minutes. After comparing the peak shape in above column with Xterra RP 18(150*4.6mm*3.5µ), in the mobile phase were 0.1% ortho phosphoric acid in water (Solvent A) and organic modifier is acetonitrile (Solvent B), the peak shape found good. The hydrophobicity of the column is distributed through the entire structure of the particle backbone, resulting in a rugged hybrid (inorganic/organic) particle that can be operated in high flow rates, high temperature and low to high pH. The surface area is 175 m^2/g , pore size is 125 A˙ and the carbon load is 15%. The optimized gradient are mentioned below

Time	A%	B%	Mode
0	30	70	Linear
5	10	90	Isocratic
7	10	90	Isocratic
10	30	70	Linear

The runtime fixed were 10 minutes with an operating flow rate is 1.2 mL/min. Injection volume for standard and sample is 10µL. The retention time of Embelin is about RT 4.9. The standard Embelin has been purchased from sigma aldrich. The ortho-phosphoric acid, Acetonitrile, Methanol were HPLC grade chemicals and water used in the experiment were collected from Purifier system milli-q. The dried Embelia ribes has been purchased from local market and authenticated.

Preparation of stock and calibration standard solutions

A stock solution of Embelin reference standard (purity 99%) was prepared by dissolving an accurately weighed 10 mg of Embelin in 100 mL methanol in a volumetric flask. From this solution various concentrations of the standard solution were prepared in 10 mL of methanol in a volumetric flask to obtain final concentrations at 150, 100, 50, 20 and 10 µg/mL.

Quantification of Embelin from Embelia ribes with different solvents

Each sample (100g) was extracted with 500 ml of different solvents like hexane, ethyl acetate, chloroform and methanol in a soxhlet apparatus for 5h. Each extract were filtered separately with whattman filter paper with suction. The filtrate was concentrated under reduced pressured at 60^0C using a rotary vacuum evaporator. Each dried extract (10 mg) was accurately weighed and transferred to a 10 mL volumetric flask. Methanol was added to the volume (final concentration 1,000 µg/ mL). Aliquot of the solution (2.5mL) was diluted with methanol in a 25 mL volumetric flask to make a concentration of 100µg/ mL. Prior to analysis, the solutions were filtered through 0.25 µm membrane filters.

Preparation of Formulation sample for analysis

Embelia ribes has been used in many herbal formulations because of its extensive medicinal properties. The formulated sample has purchased from IMCOPS, Chennai. The formulated information has collected from book “Formulary of Siddha Medicines” published by IMCOPS (The Indian Medical Practitioners Co-Operative Pharmacy and Stores Ltd). The Pharmacopoeia of siddha medicine was first published in 1956 by Dr. V. Narayanaswami and Dr. C.S Uthamaroyan.

S.No	Name of Formulation	Used for Treating	Sample ID
1	Kanthaka Rasayanam	venereal diseases, skin diseases, Leprosy.	FOR – 1
2	Inji Legiyam	Abdominal pains, vomiting and indigestion	FOR – 2
3	Meganaatha Kulikali	Contisipation with fever, intestinal worms and useful also as a purgative.	FOR -3
4	Mahaa vallaathi legiyam	Skin diseases, arthiritis, itches, ulcers and piles	FOR – 4
5	Nilaavaarai Chooranam	Gaseous distension of stomach, hiccup, vomiting, constipation and biliousness.	FOR – 5
6	Thooduvalai Ney	Asthama, bronchitis and coughs	FOR – 6
7	Thaaleesaddi Choornam	Gastritis, colic, gaseous distension of stomach and Indigestion	FOR – 7
8	Karudan Kizhangu	Skin diseases, leprosy, diseases of the hair and scalp such as alopecia.	FOR - 8
9	Abana	Moderate hypertension, cardiovascular and cerebrovascular condition requiring inhibition of platelet aggregation	FOR - 9
10	Gasex	Indigestion, dyspepsia, gaseousness	FOR -10
11	Diakof	Medication for cough	FOR – 11
12	Koflet	Bronchial mucosal irritation	FOR - 12
13	Mentat	Mental fatigue, speech disturbances and anxiety	FOR - 13

A known quantity of above formulation is weighed into a tarred 10 mL vial and soaked with Hexane. After 48hrs the solvent colour changed and it was sonicated separately for 30 minutes, and then allowed to stand for an hour. The concentrated, dried over nitrogen for 15 minutes to evaporate the residual hexane. Weighed again and prepared by adding 2 mL of methanol followed with diluting to get the concentration 100 ppm with diluent.

Method Validation

The proposed method has been validated to show some specific parameters like Limit of Detection (LOD), Limit of Quantification (LOQ), Precision, Linearity and Robustness. The Accuracy at LOQ level, shown here in terms of percent recovery of the known quantity of Embelin added to formulated sample and its recovery calculated.

Precision

The system precision has been verified by observing the %RSD of replicate injections of standard. It was verified with different day analysis to show the repeatability.

Limit of Quantification and Limit of Detection

To determine the LOD and LOQ of the proposed method, a concentration which is showing signal to noise ratio 3 for LOD and signal to noise ratio 10 has been injected. ICH describes several ways to determine the detection and quantitation limits. In the present study by observing the S/N ratio of LOD and LOQ the concentration arrived.

Linearity

Linearity was determined by using Embelin standard stock solution of 1000 µg/ mL in methanol. The solution further diluted to get 10 to 150 µg /mL of the standard solution was prepared (n = 3). The calibration graphs were obtained by plotting the peak area versus the concentration of the standard solutions and its correlation coefficient has been verified.

Robustness

The robustness of the method was determined to assess the effect of small but deliberate variation of the chromatographic conditions on the determination of Embelin. Robustness was determined by changing the pH of the buffer used in mobile phase +0.2 and -0.2 unit and its change in retention time and % RSD of standard verified.

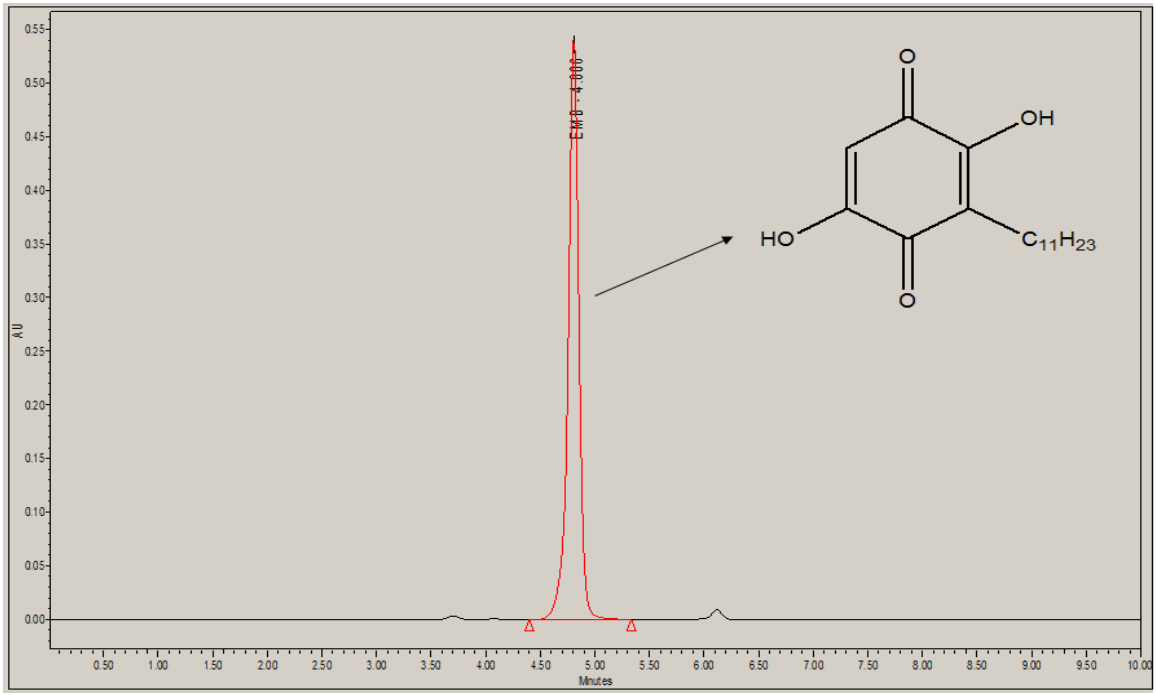
Solution Stability

The stability of the drug in solution during analysis was determined by repeated analysis of samples during the course of experimentation on the same day and also after storage of the standard solution for 15 h under refrigeration (10 ± 0.5°C).

RESULTS AND DISCUSSION

HPLC method with shorter runtime to get the analysis results faster, a method has been developed and showed with minimum validation parameter. The mixture of 0.1 % ortho phosphoric acid and acetonitrile gave optimum chromatographic separation of Embelin with the other peaks in the extract (Figure 1).

Figure 1
HPLC fingerprint of Embelin.



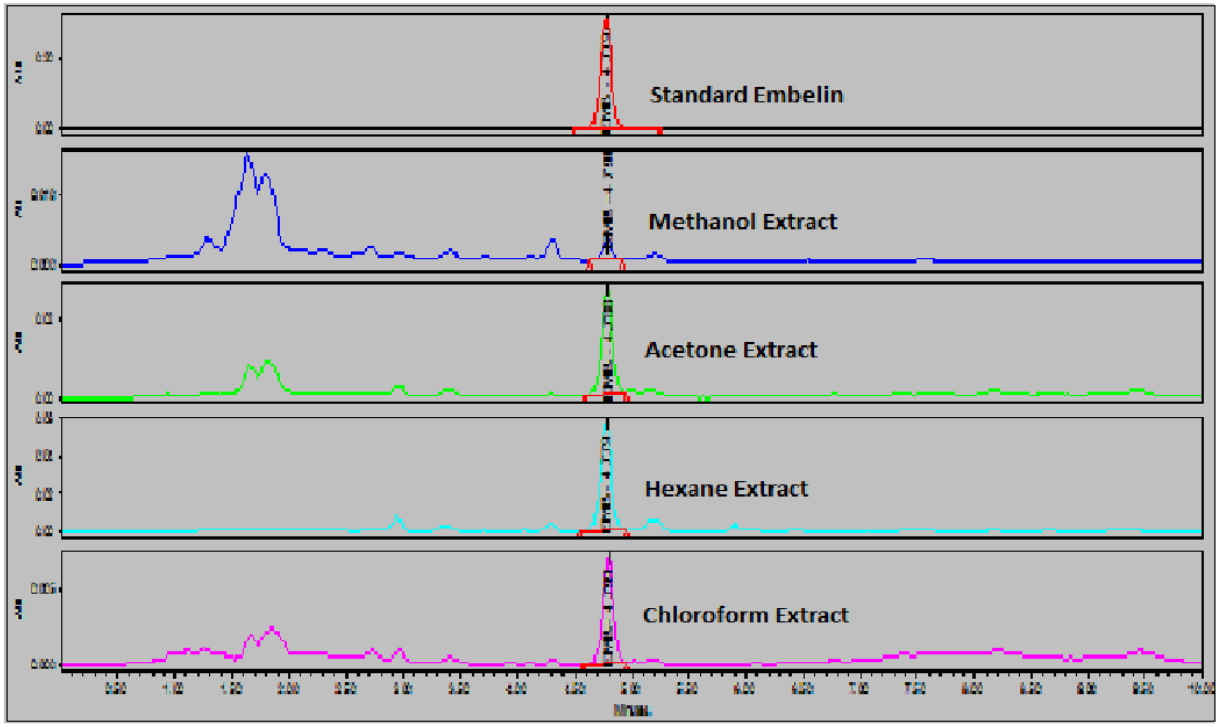
The wavelength at 280 nm was used for all measurements due to its maximum absorption. The content of Embelin in different extracts of Emblia ribes were calculated in which Hexane extract shows more content of Embelin (Table1, Figure 2).

Tables 1
Yield of crude extract and the content of Embelin in the extracts of E.ribes by the proposed HPLC method.

Name of the crude extract	Yield of crude extract*	Embelin content in crude extract*
Hexane	6.52 ± 0.21	16.42 ± 0.12
Chloroform	7.2 ± 0.53	8.3 ± 0.31
Acetone	9.4 ± 0.33	7.8 ± 0.15
Methanol	15.6 ± 0.23	3.6 ± 0.22

Expressed as mean ± standard deviation (SD; n = 3).

Figure 2
Comparison of Standard Embelin with Different extracts.



Similar HPLC condition was applied for the formulation samples and data are given in Table 2.

Table 2
Content of Embelin in Different Formulations

Sample	FOR-1	FOR-2	FR-3	FOR-4	FOR-5	FOR-6	FOR-7	FOR-8	FOR-9	FOR-10	FOR-11	FOR-12	FOR-13
% w/w	0.06	0.05	0.03	0.03	0.03	0.03	0.02	0.02	0.06	0.05	0.03	0.03	0.03

The method was validated for its linearity, precision, accuracy, LOD (0.17 µg/mL), and LOQ(0.5 µg/mL). The calibration graph for Embelin was within the concentration range of 10 – 150 µg/mL with a correlation coefficient (r^2) of 0.998 (Table 3). The interday and intraday precisions of Embelin are presented in Table 4. The results showed acceptable precision and robustness of the method with RSD values lower than 2% (Table 5), which indicate a high sensitivity of the method.

Table 3
Method validation parameters for the quantification of Embelin by the proposed HPLC method.

Parameters	**Results**
Linear range	10-150 µg/mL
Regression equation	y= 53720x + 10157
Correlation Coefficient (r^2)	0.998
LOQ (µg/mL)	0.17 µg/mL
LOD (µg/mL)	0.5 µg/mL

Table 4
Precision of Embelin determination by the proposed HPLC method..

Day	% RSD
Day-1	0.55
Day-2	0.41
Day-3	0.50

Table 5
Robustness of Embelin determination by the proposed HPLC method.

Condition	% RSD	Retention Time
pH (as Such)	0.3	4.9
Solution Stability (at 5 deg)		
After 15hrs	0.33	
Robustness		
+ Organic	0.37	5.1
- Organic	0.34	5.6
+ Flow	0.30	4.7
- Flow	0.16	5.6

CONCLUSION

A new RP-HPLC method for quantitative determination of Embelin in different extracts and in formulations is established. This Method was found to be simple, sensitive, accurate, precise and reproducible. It has been shown with excellent repeatability and it can be easily adopted for determining Embelin in herbal formulations. This proposed method will be useful for quantitative analysis in standardization and quality assessment of *Embelia ribes* extracts for pharmaceutical and cosmetic uses.

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