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Research Article

# Validation of HPTLC Method for Quantification of Embelin from *Embelia ribes* Burm. F.

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**ABSTRACT**
The study was carried out to develop a validated HPTLC method based on ICH guidelines for the quantification of embelin from medicinally important plant *Embelia ribes* Burm.F. Methanolic extract of the dried fruit of the plant was sonicated and used for the analysis. CAMAG Linomat 5 and CAMAG TLC scanner was used for HPTLC studies. Chromatography was performed on aluminium-backed silica gel 60F254 E. MERCK KGaA HPTLC plates of 250 µm thickness were used. Chloroform: ethyl acetate: formic acid (5:4:1 v/v/v) was used as the mobile phase and derivatization was carried out with anisaldehyde sulphuric acid. The method was developed and validated for specificity, linearity, range, accuracy and precision. Linearity was established in the range of 0.1 to 0.5 µg when plotted against their respective peak area. LOD and LOQ values were found to be 0.46 µg/mL and 1.39 µg/mL respectively. The External Standard Method and linearity graph was used for quantification analysis. The concentration of embelin in dried fruits of the plant is found to be 1.657µg/mg or 0.1657%. The statistical analysis conclusively establishes statistical fitness of the method. It is a specific, sensitive and reproducible HPTLC method for the quantification of embelin from *Embelia ribes* Burm.F.

**Keywords:** *Embelia ribes* Burm.F, Embelin, HPTLC, ICH, Validation, Quantification.

## INTRODUCTION

*Embelia ribes* Burm.F, is a woody climber, belonging to the family Myrsinaceae. It is popularly known as false black pepper, Vavding or Vidanga. *Embelia ribes* Burm.F grows in semi-evergreen and deciduous forests at an altitude of 1,500 m, throughout India. *Embelia ribes* Burm.F is a highly valuable medicinal plant with established anthelmintic, carminative, antibacterial, antibiotic, hypoglycemic, and antifertility properties[1]. It was identified by Susruta (Father of surgery) as anthelmintic, alternative & tonic. *Embelia ribes* Burm.F is in great demand in Ayurveda and the pharmaceutical industry leading to over harvesting and exploitation of this medicinal plant and resulting in tremendous pressure on its natural populations. *Embelia ribes* Burm.F is listed in the 'Priority Species List' for cultivation by the National Medicinal Plant Board and the Maharashtra State Horticulture and Medicinal Plant Board (MSHMPB)[2]. Sparse population and distribution of species, poor natural regeneration and unknown propagation techniques hampers the availability of 'Quality Plant Material' (QPM). Misidentification of this species along with the use of adulterants and substitutes has further aggravated the problem of this highly traded medicinal plant[3]. *Embelia ribes* Burm.F yields Embelin (embolic acid / 2, 5-dihydroxy-3-undecyl-2, 5-cyclohexadiene -1, 4-benzoquinone) (Figure 1) which is a main phytoconstituent of the plant and which along with other secondary metabolites is responsible for the wide range of its clinical applications. The quality, efficacy and establishment of authenticity of herbal drugs can be ensured by finding reliable characteristics. Determination of important phytochemical constituent or marker compound helps us to overcome this problem. Development of chemical fingerprints of such biomarkers using HPTLC is an effective tool for determining the authenticity and reliability of chemical constituent of any herbal drug and its formulation [4]. The rapid identification of such active compound or lead molecule paves the way for further investigation in the pharmaceutical industry[5]. A validated HPTLC method of quantification based on ICH guidelines ensures reproducibility of result. The characteristic pattern of chromatographic bands is easy to comprehend. The present study was aimed at development and validation of HPTLC method for quantification of embelin from *Embelia ribes* Burm.F by following ICH guidelines [6].

Synonym: 2,5-Dihydroxy-3-undecyl-2,5-cyclohexadiene-1,4-dione
Empirical Formula: $C_{17}H_{26}O_4$
Molecular Weight: 294.39

## MATERIAL AND METHODS:

*Plant Material*

Fruits of *Embelia ribes* Burm. F were collected from western ghats region of Maharashtra and authenticated at the Department of Botany, RTM University, Nagpur.

*Instrument*

**Author for Correspondence*

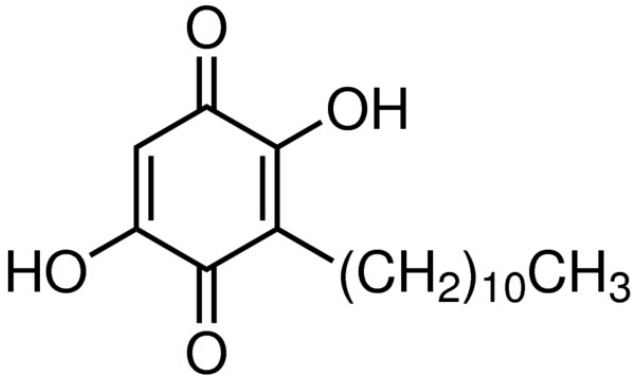


Figure 1: Structure of embelin.

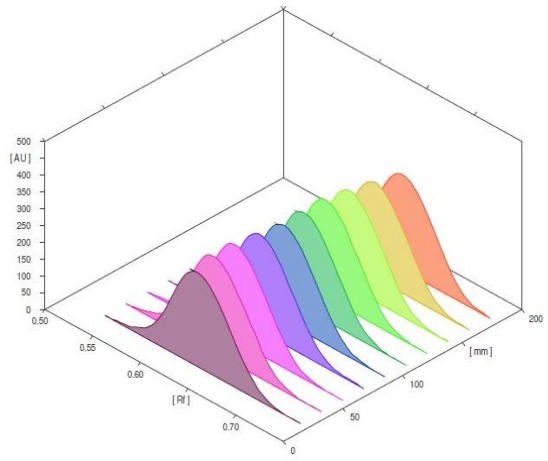


Figure 2: Densitogram of standard embelin.

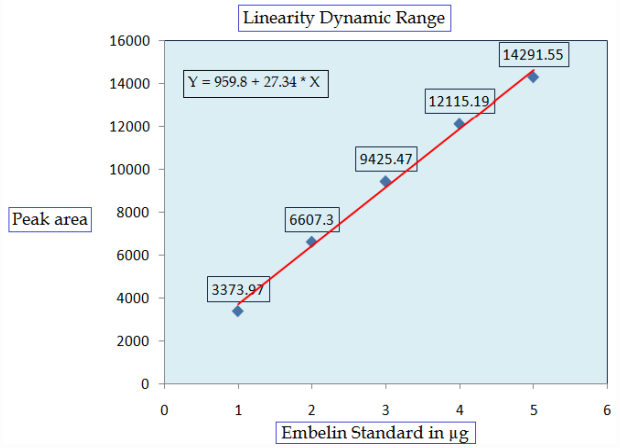


Figure 3: Linearity studies of Peak area vs embelin at various concentrations

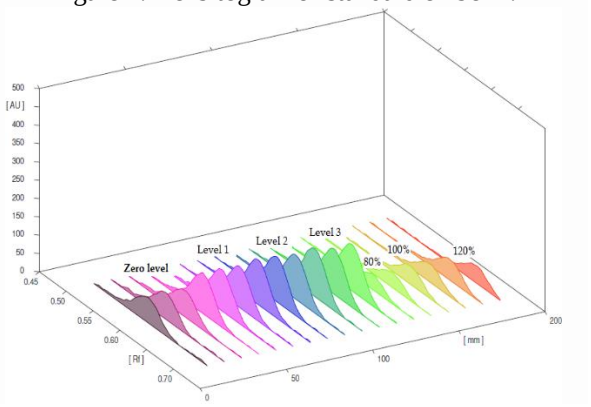


Figure 4: Densitogram showing range of embelin in fruit extract, spiked extracts and standard solutions at 80%, 100% and 120% concentration.

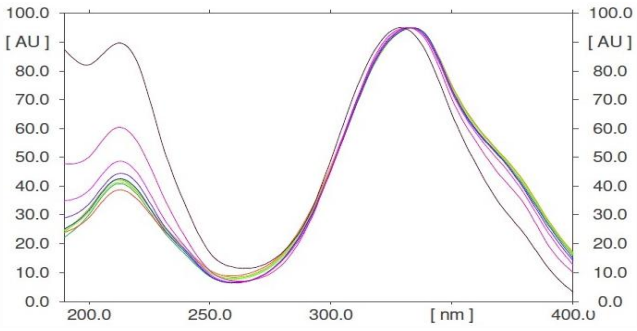


Figure 5: Overlay of embelin from range of standard solution and fruit extract

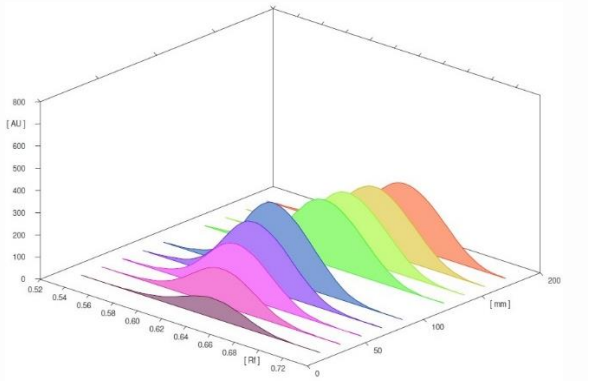


Figure 6: Densitogram of embelin from range of standard solution and fruit extract.

Camag Linomat V sample applicator, Camag Twin trough glass chamber and Camag TLC Scanner IV equipped with Cats 1.4.6 version software.

*Chemicals*

Standard embelin was sourced from Sigma Aldrich. Reagents and solvents like methanol, chloroform, ethyl acetate, formic acid were of AR grade and purchased from HiMedia.

*Preparation of Standard Stock Solution*

*Preparation of stock (A) solution of Embelin (1µg/µL)*

Stock (A) solutions of Embelin (1µg/µL) was prepared in methanol. 10.0 mg of standard Embelin was accurately weighed and transferred to a 10.0 mL standard volumetric flask. The contents of the flask were initially dissolved in 5.0 mL of methanol, followed by sonication and then diluted up to the mark with methanol.

*Preparation of stock (B) solution for Embelin (0.1 µg/µL)*

From the standard stock (A) solution, 0.1 mL was transferred to a 10.0 mL standard volumetric flask. The contents of the flask were initially dissolved in 5.0 mL of

Table 1: Chromatographic condition

| Parameters | Description |
|---|---|
| Stationary phase | Silica gel $60F_{254}$ pre-coated on aluminium sheet. |
| Mobile phase for Embelin | Chloroform: Ethyl acetate: Formic acid (5:4:1 v/v/v). |
| Prewashing of the plate | Methanol and activated at 110ºC for half an hour. |
| Development of the chamber | CAMAG Twin Trough Chamber |
| Chamber saturation | 20 min |
| Sample applicator | CAMAG LINOMAT V |
| Band length | 8 mm |
| Development distance | 80 mm |
| Derivatizing reagent | Anisaldehyde sulphuric acid |
| Drying of plate | At 110ºC for 5 min |
| Densitometric scanner | CAMAG TLC scanner IV |
| Lamp | Tungsten |
| Wavelength | 254,366 and 540 nm |
| Chromatographic evaluation | CAMAG TLC software Win cats1.4.6 |

Table 2: Rf and peak value of standard embelin.

| S. No. | Concentration of embelin | Peak area of embelin | Rf |
|---|---|---|---|
| 1. | 0.5µg | 12529.23 | 0.63 |
| 2. | 0.5µg | 12779.3 | 0.63 |
| 3. | 0.5µg | 12639.68 | 0.63 |
| 4. | 0.5µg | 12779.1 | 0.63 |
| 5. | 0.5µg | 12720.16 | 0.64 |
| 6. | 0.5µg | 12678.26 | 0.64 |
| 7. | 0.5µg | 12868.75 | 0.64 |
| 8. | 0.5µg | 12707.04 | 0.64 |
| 9. | 0.5µg | 12752.58 | 0.64 |
| 10. | 0.5µg | 12749.95 | 0.65 |
| Mean | | 12720.40 | 0.64 |
| S.D. | | 91.80 | |
| %R.S.D. | | 0.72 | |

methanol, followed by sonication and then diluted up to the mark with methanol. Thus a working stock solution of Embelin of 0.1 µg/µL was prepared in methanol.

*Preparation of samples*

Embelin is freely soluble in methanol, hence methanol was used for extraction of fruit powder during method development and validation for the plant. Accurately weight 500 mg of fruit powder of *Embelia ribes* Burm.F. was extracted with 10.0 mL of methanol. The mixture was sonicated for 30 min and it was kept overnight for extraction. It was filtered through Whatmann filter paper No. 41 and filtrate was subjected to HPTLC for quantification of embelin. Plant extracts of the concentration 50 µg/µL was prepared.

*Method development*

Chromatogram was developed for Embelin by selecting the mobile phase after trying few combinations of solvents. The best resolution was observed in the selected solvent system of Chloroform: Ethyl acetate: Formic acid (5:4:1 v/v/v). The chromatography chamber was saturated for 20 min. for the optimum result. The developed HPTLC plate was dried at 105 ºC and derivatized with anisaldehyde sulphuric acid reagent. The derivatized plate was again heated for easy identification of compact bands. Densitometric analysis was performed at absorption maxima of wavelength 254, 366 and 540 nm (Table 1).

Table 3: Rf values and Peak area of embelin at various concentrations.

| S. No. | Concentration of embelin | Peak area of embelin | Rf |
|---|---|---|---|
| 1. | 100 ng | 3373.97 | 0.64 |
| 2. | 200 ng | 6607.37 | 0.63 |
| 3. | 300 ng | 9425.47 | 0.63 |
| 4. | 400 ng | 12115.19 | 0.63 |
| 5. | 500 ng | 14291.55 | 0.62 |

*Method Validation*

The method was developed and validated for specificity, linearity, range, accuracy, precision, limits of detection (LOD) and limit of quantification (LOQ) as per the ICH guidelines.

*Specificity*

Selectivity and specificity assay of embelin was done by running successive 10 bands of 0.5µg concentration of Embelin to identify the presence of standard and to test its accuracy in term of peak area in all bands.

*Linearity*

A linear relationship was evaluated by dilution of standard stock solution in the range of 0.1 µg to 0.5 µg. Calibration curve was plotted for the range of embelin against its peak area to establish the linearity. The correlation coefficient, y-intercept, slope of the regression line was determined.

*Range and Accuracy*

The range was studied by spiking the dose of 80%, 100% and 120% embelin to the seed extract and observing its effect on the chromatogram. ICH guidelines suggested the study of chromatogram +/-20% over the range.

*LOD and LOQ*

Sensitivity was determined by establishing the limit of detection (LOD) and limit of quantitation (LOQ). As per the ICH guidelines, Standard deviation (SD) of response and slope was calculated for LOD (DL=3.3xSD/S) and LOQ (DL=10xSD/S).

*Precision studies*

*Intra-day Precision or Repeatability*

As per the ICH guidelines similar bands (each 5 µL) of standard Embelin solutions (0.1 µg/µL) were run on a HPTLC plate on different time of the day, the densitograms and peak areas were recorded to study Intra Day precision.

*Inter-day Precision*

Inter-day precision was performed by recording peak areas of standard Embelin solutions applied for three consecutive days. Mean, SD and %RSD was calculated to support the experiment.

*Quantification of Embelin*

Quantification was carried out as per the ICH guidelines by using the regression equation. The linear regression graph of range of standard Embelin applied on plate is

Table 4: Peak area of range of embelin in fruit and spiked extracts at 80%, 100% and 120% concentration.

| S. No. | Quantity of Extract | Rf | Peak area of Embelin | Mean | SD | RSD |
|---|---|---|---|---|---|---|
| 1. | 0.4 µg | 0.60 | 2458 | 2501.3 | 39.11 | 1.56 |
| 2. | 0.4 µg | 0.60 | 2512 | | | |
| 3. | 0.4 µg | 0.60 | 2534 | | | |
| 4. | Sample +80% | 0.60 | 3753 | 3776.3 | 32.93 | 0.87 |
| 5. | Sample +80% | 0.61 | 3762 | | | |
| 6. | Sample +80% | 0.61 | 3814 | | | |
| 7. | Sample +100% | 0.61 | 4206 | 4187.3 | 18.04 | 0.43 |
| 8. | Sample +100% | 0.61 | 4186 | | | |
| 9. | Sample +100% | 0.62 | 4170 | | | |
| 10. | Sample +120% | 0.62 | 4512 | 4450.3 | 86.07 | 1.93 |
| 11. | Sample +120% | 0.62 | 4352 | | | |
| 12. | Sample +120% | 0.62 | 4487 | | | |

Table 5: Precision studies for embelin.

| S. No. | Quantity of Embelin | Peak area of Embelin | | |
|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 |
| 1. | 0.5µg | 12529.23 | 13217.98 | 13221.53 |
| 2. | 0.5µg | 12779.3 | 13411.84 | 13416.97 |
| 3. | 0.5µg | 12639.68 | 13230.62 | 13235.03 |
| 4. | 0.5µg | 12779.1 | 13284.48 | 13382.76 |
| 5. | 0.5µg | 12720.16 | 13358.96 | 13408.91 |
| 6. | 0.5µg | 12678.26 | 13239.64 | 13434.29 |
| 7. | 0.5µg | 12868.75 | 13421.10 | 13360.23 |
| 8. | 0.5µg | 12707.04 | 13315.69 | 13290.89 |
| 9. | 0.5µg | 12752.58 | 13503.71 | 13489.29 |
| 10. | 0.5µg | 12749.95 | 13313.02 | 13302.12 |
| Mean | | 12720.40 | 13329.70 | 13354.20 |
| S.D. | | 91.80 | 93.78 | 88.80 |
| %R.S.D. | | 0.72 | 0.70 | 0.66 |

Table 6: Quantification of embelin showing peak area and Rf values.

| S. No. | Quantity of Extract | Peak area of Embelin | Rf |
|---|---|---|---|
| 1. | 0.4 µg | 12928.67 | 0.64 |
| 2. | 0.4 µg | 12886.99 | 0.63 |
| 3. | 0.4 µg | 13081.14 | 0.63 |
| 4. | 0.4 µg | 12702.62 | 0.63 |
| | Mean | 12898.63 | 0.63 |
| | S.D. | 155.73 | |
| | %RSD | 1.21 | |

calculated and is used for the quantification of Embelin in fruit extract.

## RESULT AND DISCUSSION

*Method Validation*

*Specificity*

The method was found to be specific and selective for the embelin (Fig.2). Assay was done by using 10 bands of standard solution, CV was found to be 0.72 which is well below the statistically accepted level of 2. (Table 2)

*Linearity*

Minimum 5 concentrations were studied as per the ICH guidelines for establishing linearity. A linear relationship was obtained for successive dilution of standard stock solution in the range of 0.1 µg to 0.5 µg. Calibration curve was plotted for the range of embelin against its peak area which exhibited linearity. Linear equation of Y=959.8+27.34X was obtained where 'Y' is the peak area of Embelin and 'X' is the concentration of standard stock solution. SD of 3.82 and coefficient of correlation of 0.997 establishes statistical fitness of linearity studies (Fig. 3, Table 3).

*Range and Accuracy*

The peak area responded well when fruit extract (zero value) was spiked by 80% (level 1), 100% (level 2) and 120% (level 3) embelin. The accuracy in terms of area and recovery of embelin is not affected by more than 10% when extract was spiked +/-20% over the specified range. (Fig. 4). The peak area over the range shows RSD values less than 2 (table 4)

*Limit of Detection (LOD) and Limit of Quantitation (LOQ)*

The detection limit (LOD) and quantitation limit (LOQ) were calculated on the basis of Standard Deviation of the Response and the Slope as per the ICH guidelines. LOD and LOQ were determined by using equations, LOD= 3.3 * σ/slope and LOQ= 10 * σ/slope, Where, σ = Standard Deviation, Slope = Slope of the calibration curve

LOD was found to be 0.46 µg/mL and LOQ was found to be 1.39 µg/mL

*Precision Studies*

*Intra-day Precision or Repeatability*

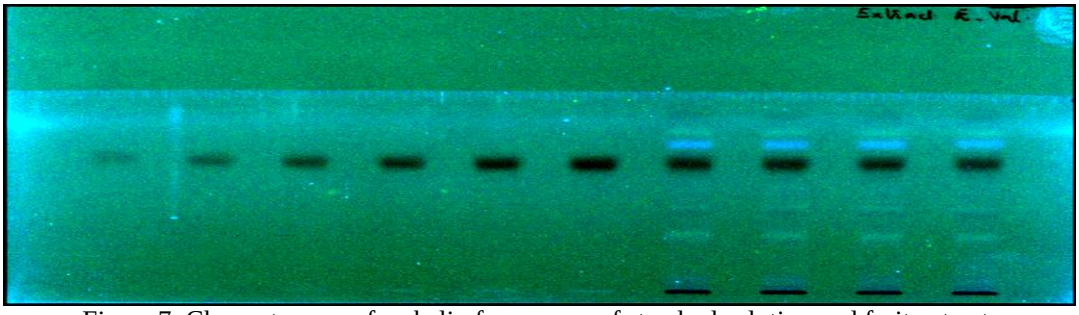

Figure 7: Chromatogram of embelin from range of standard solution and fruit extract.

As per the ICH guidelines similar bands (each 5 µL) of standard Embelin solutions (0.1 µg/µL) were run on a HPTLC plate on different time of the day, the densitograms and peak areas were recorded. The mean, standard deviation, and coefficient of variation [%] were calculated for peak area and $R_F$. The HPTLC profile obtained after derivatization show the analyte eluted to the same distance thus showing same $R_f$ in all the samples loaded on the plate**.** The results obtained from study of spotting repeatability/instrument precision are listed in Day 1 of Table No. 5. The (RSD) of the peak areas for each loading of Embelin is a much less than 2 which indicates more reliability of the results.

*Interday Precision*

The peak areas of standard embelin were recorded for three consecutive days. The values of mean peak area, standard deviation and related standard deviation were calculated for standard embelin on three different days. The results are given in Table 5. As the values of % relative standard deviation of the peak areas of embelin for all sample solutions are below 2 it shows that the method is precise for performing the analysis.

*Quantification of embelin*

Quantification was carried out as per the ICH guidelines by using the regression equation. Linear equation was obtained for embelin from range of standard solution and fruit extract (Fig. 3,5,6 and 7). Mean peak area of Extract is 12899.86 for 4 µl of extract (Table 6). Calculations for the assay of Embelin, in 500 mg dried fruit powder of *Embelia ribes* Burm.F was carried out using the equation $Y = 27.34 * X + 959.8$ obtained during linearity studies. According to the calibration curve, the concentration of Embelin was found to be 1.657µg/mg or 0.1657%.

## CONCLUSION

Validated HPTLC method has been developed for identification and quantification of Embelin from methanolic extract of *Embelia ribes* Burm.F. It can be used for rapid identification, authentication, quality control analysis and quantitative evaluation of embelin from *Embelia ribes* Burm.F. Embelin was found linear in the range of 0.1 µg to 0.5 µg. It is a specific, sensitive and reproducible method for the quantification of embelin from *Embelia ribes* Burm.F. Keeping in mind the wide therapeutic application of embelin such method will ensure establishment of 'Quality Plant Material' (QPM) for herbal dependent industry and to counter the menace of adulteration to a greater extent.

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