ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS
ESTIMATION OF EMBELIN AND CURCUMIN IN BULK AND POLYHERBAL
FORMULATION.

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Abstract: The present manuscript describes simple, rapid, accurate, precise and economical RP-HPLC method for the simultaneous estimation of Embelin and Curcumin in Bulk powder mixture and Polyherbal formulation. The method was successfully applied to Polyherbal Formulation because no interference from other constituents was found. The suitability of this method for the quantitative determination of Embelin (EMB) and Curcumin (CUR) was proved by validation. The developed method show best result in terms of linearity, accuracy, precision, Limit of detection (LOD), Limit of Quantitation (LOQ). The linearity range of Embelin and Curcumin were found to be 5-25 μg/ml and 25-125 μg/ml respectively. The method showed good regression r²= 0.999 and recovery were found in the range of 98 – 102% for both Embelin and Curcumin. The proposed method was found to be simple and sensitive for the routine quality control application of Embelin and Curcumin in bulk and Polyherbal Formulation. The results of analysis have been validated statistically and by recovery studies.

Keywords: Embelin(EMB); Curcumin(CUR); Embelia ribes; Curcuma longa; Recovery; RP-HPLC method; Method Validation.

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INTRODUCTION

Since ancient times, the drug 'Vidanga' or 'Baibidanga' has been an important ingredient in a number of Ayurvedic formulations. 'Vidangh' is the dried berries of fruits of E. ribes belonging to family Myrsinaceae. Embelia ribes is most widely used in Indian traditional medicine. The fruits of Embelia ribes reported to contain mainly benzoquinone derivatives such as Embelin (2, 5-dihydroxy-3-undecyl-2, 5- cyclohexadiene-1, 4- benzoquinone). The plant Embelia ribes contains embelin, quercitol, and fatty ingredients; an alkaloid, christembine, a resinoid, tannins and minute quantities of a volatile oil.\[1,2\]

Vidanga fruits contain 1.85-2.15% w/w of Embelin. Embelin is Soluble in DMSO, alcohol, benzene chloroform and Insoluble in water.\[3\] The dried fruit has been used in India since ancient times as an anthelmintic. Embelia ribes has been shown to possess astringent, carminative, stimulant, antioxidant, antispermetogenic, anti-bacterial and anticancer activity.\[4\] It has been used as a cosmetic agent to cure skin disorders for centuries. E. ribes is used especially for dyeing hairs, good pimple remover, treating acne, treating carbuncle infections, treating vitiligo and leucoderma, abdominal disorders, lung diseases, constipation, indigestion, fungus infection, mouth ulcer, sore throat, heart disease and obesity. \[5, 6\]

Curcumin is a polyphenolic compound [(1E,6E)-1,7-bis(4-hydroxy-3 (methoxyphenyl)-1,6 heptadiene3,5dione)] derived from Turmeric. Turmeric consists of dried and fresh rhizomes of the plant known as curcuma longa Linn belonging to family zingiberaceae. Traditionally, the plant Curcuma longa widely used as a gold-coloured spice in Indian subcontinent which impart flavour, colour to the food, act as a medicinal herb and used in cosmetic, and textile industry as a dye. Curcumin is the active ingredient of turmeric, which is used daily in Indian and other South Asian countries as a spice. Turmeric has been found to be a rich source of phenolic compound. The colouring principle of turmeric was isolated in the 19th century and was named curcuminoids. All curcuminoids are often referred to simply as “Curcumin”. Three curcuminoids are isolated from Turmeric viz; curcumin I (94%), Curcumin, II (demethoxycurcumin-6%), curcumin III (bisdemethoxycurcumin-0.3%). \[7, 8\]

Turmeric contains 2.85% to 6.14%w/w Curcumin. Curcumin is freely soluble in methanol, chloroform, ethanol, Acetone and practically insoluble in water. Curcumin has the ability to suppress both acute and chronic inflammation as it blocks the formation of cyclo-oxygenases (COX-2) and other enzymes involved in inflammation. Turmeric Vanishing Cream helps in preventing the damage of the skin from UV rays of the sun, and thus maintains the original colour of the skin with enhancing the appearance of the skin. \[9\] The effects of administration of Embelin and Curcumin on lipid peroxidation, hepatic glutathione antioxidant defense and hematopoietic cells were examined during hepatocarcinogenesis and also have
chemoprotective action. Beneficial effects of Embelin and Curcumin were found against oxidative tissue damage during chemically-induced hepatocarinogenesis in rats.[10]

![Figure 1: Structure of Embelin](image1.png)  ![Figure 2: Structure of Curcumin](image2.png)

Literature survey revealed that the Spectrophotometry method, High pressure liquid chromatography (HPLC) and High performance thin layer chromatography (HPTLC) methods are available for estimation of Embelin isolated from Embelia ribes and Polyherbal Formulation. And also analytical methods such as High pressure liquid chromatography, High performance thin layer chromatography and Spectrophotometry methods are available for estimation of Curcumin isolated from Curcuma Longa and Herbal formulation. To the best of our knowledge, there is no analytical method available for simultaneous estimation of Embelin and Curcumin in bulk and Polyherbal Formulation.

**MATERIALS AND METHODS**

**Chemical and Reagents:** Herbal Standards of Embelin and Curcumin was provided by Aum Research Laboratory, Gandhinagar. Polyherbal Formulation containing Embelin and Curcumin and Samples of Curcuma longa and Embelia ribes were procured from local market, Ahmedabad. All the chemicals used were of AR grade obtained from Finar Chemicals Ltd, Ahmedabad.

**Apparatus:** Ultrasonicator (Ultrasonic bath, Popular India), pH meter (HI 2215, Hanna instrument), UV Spectrophotometer (UV 2080 Plus, Analytical Technologies), HPLC analysis was performed on HPLC 3000, Analytical Technologies Pvt. Ltd (Software: Clarity) using UV 3000 Detector and Quaternary Pump-P 3000 EDC analyze HPLC Pansystem.

**Chromatographic Condition:** The chromatographic separation was performed on Merck Kromasil C18 column (250 mm × 4.6mm, 5 μm particle size). The mobile Phase Methanol : 0.1% Trifluoroacetic acid in Water (88:12 v/v) pH 2.5 were selected with 1 ml/min flowrate. The injection volume was 20μl. The detection Wavelength was selected 288 nm.

**Preparation of standard stock solutions:** Accurately weighed quantity of 20mg of Embelin and Curcumin were transferred into 10 ml volumetric flasks, dissolved with shaking the flasks and diluted up to mark with Methanol. Thus a stock solution having strength of 2000 µg/ml were
Prepared. From above stock solution, further dilution were made to get 200 µg/ml solution of Embelin and Curcumin.

**Preparation of Extract of Polyherbal formulation:** Twenty capsules were weighed and average weight of content was determined. The powder equivalent to 557mg of Curcuma longa powder was transferred into a 50 ml volumetric flask and water was added up to the mark. This solution was macerated overnight at least 24 hours. Then it was filtered using Whatman filter paper. Residue was washed with fresh water 2-3 times. Then residue was dissolved in 25 ml methanol in volumetric flask. The solution was sonicated for 30 min then filtered it and diluted up to the mark with methanol. From above Solution 5 ml was taken and diluted up to 25 ml with methanol to prepare 200 µg/ml Curcumin solution. A sample solution was injected under chromatographic condition for further analysis.

**Preparation of Extract from Bulk Powder Mixture of Embelia ribes and Curcuma longa:** 250 mg Embelia ribes and 557 mg Curcuma longa powder was weighed and transferred in 25 ml volumetric flask. Extraction was carried out with methanol at 60 to 70°C for 30 min. The solution was filtered and volume was make up to the 25 ml with methanol. The aliquot of 5 ml of this solution was pipette out and diluted to 25 ml with methanol to give a bulk Powder mixture solution having strength of 40µg/ml of Embelin and 200µg/ml of Curcumin.

**Preparation of Binary mixture of Embelin and Curcumin:** From 200 µg/ml solution of Embelin, pipette out 6.25 ml solution in 50 ml of volumetric flask, to the same volumetric flask pipette out 31.25 ml standard solution of Curcumin (200 µg/ml), methanol was added and allow it to sonicate for 5 min. Then diluted it upto mark with methanol to give a binary mixture solution having strength of 25µg/ml of Embelin and 125µg/ml of Curcumin.

**Determination of wavelength for maximum absorbance:** The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. Solution of Embelin (10µg/ml) and Curcumin (10µg/ml) were separately prepared in methanol. Solutions were scanned between 400-800 nm. At 288 nm both drugs give good peak height and Resolution. So, 288 nm was selected for simultaneous estimation of Embelin and Curcumin in bulk and Polyherbal Formulation.
METHOD VALIDATION:

The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines.

**Linearity** Calibration curve of Embelin and Curcumin consists of different concentrations of binary mixture solution of Embelin and Curcumin ranging from 5-25 μg/ml and 25-125 μg/ml. The solutions were injected automatically through autosampler (20 μl) and chromatograms were recorded. Then calibration curves were constructed by plotting peak area against concentration of the drug, two separate calibration curve was constructed for both the drugs. Each response was the average of three determinations.

**Precision:** Precision of the method was verified by repeatability, intraday and interday precision studies. Repeatability precision were performed by repeated analysis of binary mixture containing 10 μg/ml of Embelin and 50 μg/ml of Curcumin six times. Intra-day precision was performed by analysis of binary mixture containing 5 & 25 μg/ml, 10 & 50 μg/ml, 15 & 75 μg/ml of Embelin and Curcumin respectively on the same day. Inter-day precision was performed on these three concentrations on three different days. Peak areas were expressed in terms of standard deviation (S.D.) and relative standard deviation (%RSD).

**Specificity:** Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Typically, these might include impurities, degrades etc. The chromatogram of blank was compared with those acquired from Embelin and Curcumin standards, correlation in terms of interference at retention time and peak area was evaluated to indicate the specificity of method.

**Limit of Detection (LOD) and Limit of Quantitation (LOQ):** The LOD and LOQ were estimated from the set of 3 calibration curves used to determine method linearity. The LOD and LOQ were calculated as;
LOD = 3.3 x (SD / Slope)
LOQ = 10 x (SD / Slope)

Where, SD = the standard deviation of Y-intercept of 3 calibration curves.

Slope = the mean slope of the 3 calibration curves.

**Accuracy (recovery study):** The accuracy of the method was determined by calculating recovery of Embelin and Curcumin at different spiked level by standard addition method. Standard Embelin and Curcumin solutions were added at three different levels (50, 100, 150%). At each level three determinations were performed. The amounts of standard recovered were calculated in terms of %recovery and %RSD for Polyherbal Formulation and bulk powder mixture.

**Robustness:** Robustness of the method was determined by small, deliberate changes in flow rate, pH of mobile phase and detection wavelength. Typical changes include flow rate changed to 1.0 ± 0.1 ml/min, pH of the mobile phase changed to 2.5 ± 0.1 and detection wavelength changed to 288 ± 1 nm.

**Quantitative analysis of Embelin and Curcumin in Formulation and bulk mixture of Curcuma longa and Embelia ribes:** The proposed RP-HPLC method was applied to analyze Embelin and Curcumin in Polyherbal Formulation and Powder mixture. Sample solutions of 100µg/ml Curcumin and 20µg/ml of Embelin were injected under chromatographic condition and peak areas were measured and % assay were calculated from regression equation.

**RESULT AND DISCUSSION**

HPLC Analysis: Chromatographic conditions were optimized to achieve the good resolution and peak shape for Embelin and Curcumin.

**Table 1: Chromatographic Condition**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Optimized Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Merck Kromasil C18 column (250 mm x 4.6mm, 5 µm particle size).</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Methanol : 0.1% Trifluoroacetic acid in Water (88:12 v/v) pH 2.5</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>UV Detection</td>
<td>288 nm</td>
</tr>
<tr>
<td>Injection</td>
<td>20 µl</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
</tbody>
</table>
Method validation

Linearity plots and detection limits: The plots of peak area versus respective concentrations of Embelin and Curcumin were found to be linear in the concentration range of 5-25 μg/ml and 25-125 μg/ml with correlation coefficient 0.999 and 0.999 respectively. The linearity of calibration graphs and adherence of the system to Beer’s law validated by determining correlation coefficients and SD values which were found to be well within accepted limit. The LOD were found to be 0.4 μg/ml and 2.31 μg/ml for Embelin and Curcumin respectively. The LOQ were found to be 1.24 μg/ml and 7.01 μg/ml for Embelin and Curcumin respectively.

Table 2: Calibration Curves, Limit of Detection and Limit of Quantitation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Embelin</th>
<th>Curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity Range*</td>
<td>5-25 μg/ml</td>
<td>25-125 μg/ml</td>
</tr>
<tr>
<td>Correlation Co-efficient</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>Slope</td>
<td>5717.7</td>
<td>1725</td>
</tr>
<tr>
<td>Intercept</td>
<td>-8832.1</td>
<td>-3918.3</td>
</tr>
<tr>
<td>LOD*</td>
<td>0.4 μg/ml</td>
<td>2.31 μg/ml</td>
</tr>
<tr>
<td>LOQ*</td>
<td>1.24 μg/ml</td>
<td>7.01 μg/ml</td>
</tr>
</tbody>
</table>

*Results are mean of three determinations

Precision: The %RSD value for repeatability was found to be less than 2 (n=6). Intraday and interday precision for RP-HPLC method were measured in terms of % RSD. The average percentage RSD for intraday and interday precision was found to be 0.840% and 0.852% for
Embelin. The average percentage RSD for intraday and interday precision was found to be 1.156% and 0.879% for Curcumin. The values confirm the method was precise. The results obtained are shown in Table 3.

Table 3: Results of Precision of proposed method

<table>
<thead>
<tr>
<th>Conc. (μg/ml)</th>
<th>%RSD Intraday</th>
<th>%RSD Interday</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embelin</td>
<td>Curcumin</td>
<td>Embelin</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>1.03</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>0.68</td>
</tr>
<tr>
<td>15</td>
<td>75</td>
<td>0.80</td>
</tr>
<tr>
<td>Average</td>
<td>0.840</td>
<td>1.156</td>
</tr>
</tbody>
</table>

Specificity: The chromatogram of blank was compared with those acquired from Embelin and Curcumin standards, correlation was good (in terms of t<sub>R</sub> and area) indicates the specificity of method.

Robustness: The method was found to be robust as the results were not significantly affected by slight variation in pH of mobile phase, Flow rate and Detection Wavelength.

Accuracy (Recovery study)

The average recoveries of the Embelin and Curcumin were in the range of 98 -102%. Satisfactory recoveries with small % relative standard deviations (less than 2) were obtained which indicate the method was accurate. The result of recovery study of drugs is shown in Table 4.

Table 4: Accuracy of Embelin and Curcumin

<table>
<thead>
<tr>
<th>Samples</th>
<th>%Recovery*</th>
<th>50%</th>
<th>100%</th>
<th>150%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embelin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Powder mixture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embelin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Results are mean of three determinations
Quantitative Analysis of Embelin and Curcumin in Polyherbal Formulation and Powder mixture

The developed method was applied on formulation and powder mixture; there was no interference from other components present in formulation. The concentration of Embelin and Curcumin in formulation and powder mixture were calculated by measuring their peak areas and comparing their peak areas of standard drug solution. Results are shown in Table 5.

Table 5: Quantitative analysis of Embelin and Curcumin from Polyherbal Formulation and Powder Mixture.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>%purity*</th>
<th>Content* (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EMB</td>
<td>CUR</td>
<td>EMB</td>
</tr>
<tr>
<td>Formulation</td>
<td>20</td>
<td>100</td>
<td>99.79%</td>
</tr>
<tr>
<td>Powder Mixture</td>
<td>20</td>
<td>100</td>
<td>100.87%</td>
</tr>
</tbody>
</table>

*Results are mean of three determinations

Figure 5: Chromatogram of Formulation

Figure 6: Chromatogram of Powder Mixture
CONCLUSION

Ayurvedic and herbal formulations are Polyherbal in nature. Considering the widespread use of Curcumin and Embelin in plant based medicines, a HPLC method was developed for simultaneous estimation of Embelin and Curcumin in presence of other plant constituents. The present study shows that the method developed for the simultaneous estimation of Embelin and Curcumin was simple, rapid, specific, linear, accurate, precise and robust. Hence, above said method can be successfully applied for routine quality control analysis and estimation of Embelin and Curcumin in bulk and Polyherbal formulation.

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