

# Development of response surface methodology for optimization of extraction parameters and quantitative estimation of embelin from *Embelia ribes* Burm by high performance liquid chromatography

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## ABSTRACT

**Background:** *Embelia ribes* Burm is widely used medicinal plant for the treatment of different types of disorders in the Indian traditional systems of medicine. **Objective:** The present work was aimed to optimize the extraction parameters of embelin from *E. ribes* fruits and also to quantify embelin content in different extracts of the plant. **Materials and Methods:** Optimization of extraction parameters such as solvent: drug ratio, temperature and time were carried out by response surface methodology (RSM). Quantitative estimation of embelin in different extracts of *E. ribes* fruits was done through high performance liquid chromatography. **Results:** The optimal conditions determined for extraction of embelin through RSM were; extraction time (27.50 min), extraction temperature 45°C and solvent: drug ratio (8:1). Under the optimized conditions, the embelin yield (32.71%) was equitable to the expected yield (31.07%,  $P > 0.05$ ). These results showed that the developed model is satisfactory and suitable for the extraction process of embelin. The analysis of variance showed a high goodness of model fit and the accomplishment of the RSM method for improving embelin extraction from the fruits of *E. ribes*. **Conclusion:** It is concluded that this may be a useful method for the extraction and quantitative estimation of embelin from the fruits of *E. ribes*.

**Key words:** *Embelia ribes*, response surface methodology, reverse phase-high performance liquid chromatography, ultrasonic-assisted extraction

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## INTRODUCTION

*Embelia ribes* Burm (Myrsinaceae) commonly called as false pepper is a valuable medicinal plant included in Indian system of medicine. It is a woody shrub that is used in Unani system of medicine for various ailments

and also forms a part of 75 Ayurvedic preparations.<sup>[1]</sup> It is known to occur in various parts of India and Pakistan.<sup>[2]</sup> In India, this plant abundantly occurs in the Western Ghats of Tamil Nadu and Karnataka. In lower proportions, Kerala is also a hometown for the plant.<sup>[1]</sup> The plant is known to possess antifertility, anthelmintic, carminative, antibacterial, hepatoprotective, neuroprotective, antifungal, analgesic, hypoglycaemic, antioxidant, anticancer, anticonvulsant, wound healing, adaptogenic, cardioprotective, etc., activities.<sup>[1,3,4]</sup> The seeds of the plant are used for treatment of leprosy, liver diseases and ringworm infections. Root extract is used as contraceptive and in lactation problem, anorexia, oedema, hepatitis, piles, diabetes, vitiligo, etc.<sup>[4]</sup>

*Embelia ribes* contain a number of secondary metabolites of which embelin (2,5-dihydroxy-3-undecyl-p-benzoquinone)

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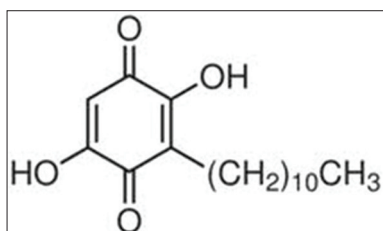
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is a major constituent [Figure 1]. About 4.33% of embelin occurs in the berries of *E. ribes*.<sup>[4,5]</sup> Embelin has been reported to possess anti-estrogenic, anti-spermatogenic, anti-inflammatory, anti-helminthic,<sup>[2]</sup> anti-diabetic<sup>[6]</sup> and wound healing<sup>[2,7]</sup> properties.

Various conventional methods are used for the extraction of phytoconstituents from plant materials, which are based on utilization of heat and agitation to enhance solubility and the rate of mass transfer. Such methods include maceration, digestion, percolation, soxhlet and heat reflux techniques. However, these methods are time and solvent consuming. Furthermore, such methods are not suitable for thermolabile drugs. Certain novel techniques are available for extraction like an ultrasound ultrasonic-assisted extraction (UAE), which can overcome some of the above stated issues. UAE is an advanced extraction technique, which is simple, inexpensive, efficient and environment friendly and also saves time and solvents.<sup>[8]</sup> The mechanism involved behind ultrasound assisted extraction is the breakdown of cell walls, decrease in particle-size and increase in the mass transfer of contents of the cells into the solvent.<sup>[8-10]</sup>

Response surface methodology (RSM) is a recent sophisticated statistical approach or a technique commonly used for optimizing a process when many factors and their interactions affect the response. This technique was developed by Box and Wilson in 1951.<sup>[11,12]</sup> This mathematical technique enables designing of experiments, where the output variables are analyzed, which is influenced by a number of input variables. RSM helps to overcome problems associated with traditional optimization methods. It helps to reduce the number of experimental trials and hence it is a time saving technique.<sup>[11,13]</sup> This technique has been enormously employed for optimization of extraction processes of various phyto-constituents like colchicine from *Gloriosa superba* tubers,<sup>[11]</sup> anthraquinones from *Rheum palmatum*,<sup>[14]</sup> anthocyanins from black currants,<sup>[15]</sup> phenolic compounds from wheat,<sup>[16]</sup> and in an UAE of total polyphenols, total tannins and *Epigallocatechin gallate* contents from *Stryphnodendron adstringens* (Mart.) Coville bark extracts were determined by Sousa *et al.* Different



**Figure 1:** Chemical structure of embelin

extraction parameters viz. ethanolic strength (% v/v), extraction time (min) and liquid to solid ratio (mg/mL) were optimized with a Box–Behnken Design (BBD) using RSM. The results demonstrated the viability of ultrasound-assisted extraction using RSM.<sup>[17]</sup> Many more studies also reported the same technology.

The current study was designed to optimize the extraction parameters of embelin from *E. ribes* through ultrasound assisted extraction. Parameters optimized includes drug: solvent ratio (g/50 mL), temperature (°C) and time (minutes).

## MATERIALS AND METHODS

### Plant material

The fruits of *E. ribes* were purchased from Yucca Enterprises Mumbai, India and authenticated by a taxonomist, Botany Department, Faculty of Science, Jamia Hamdard (Hamdard University), India. The fruits were then grounded and passed through sieve no 14 and stored in an air tight container until further utilization.

### Extraction of embelin

Six different solvents were used for the extraction of embelin from *E. ribes*, viz. methanol, chloroform, acetone, ethyl acetate, hexane, and diethyl ether. Extraction was done taking 50 mL of solvent for 10 g of the drug through sonication (TOSCHON, SW7) for 15 min at 30°C followed by quantification of embelin in different extracts.

### Single factorial experiments

After determination of the best solvent among the six investigated solvents for embelin extraction, single factorial experiments were conducted to evaluate the effect of a particular parameter on embelin extraction. Here, one parameter was varied in a particular range and the other two were kept constant. The ranges assessed for different parameters are presented in Table 1.

### Optimization of extraction parameters of embelin

The RSM was used to optimize the extraction parameters for embelin extraction techniques using Design-expert software, Stat-Ease, Inc. USA (Version 8.0.6.1), while BBD was employed in this regard. All experimental

**Table 1: Ranges of different parameters assessed in single factorial experiments**

Parameter	Lower range	Higher range
Solvent to drug ratio (mL/g)	5	20
Temperature (°C)	30	60
Time (min)	15	40

design consisted of twelve factorial experiments, and five simulates of the center point. A second-order quadratic model was used to predict the optimal point through correlation of independent variables (solvent to drug ratio, temperature, and time) and the response (percentage of embelin). The coded and the actual values of three variables are given in Table 2 and the 17 runs of BBD experimental planning is given in Table 3. The equation for three factors is:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2$$

Where Y = predicted response

$\beta_0$  = Model constant

$\beta_1, \beta_2, \beta_3$  = Linear coefficients

$X_1, X_2, X_3$  = Independent variables

$\beta_{12}, \beta_{13}, \beta_{23}$  = Cross product coefficient

$\beta_{11}, \beta_{22}, \beta_{33}$  = Quadratic coefficient.

**Table 2: Coded and actual levels of three variables**

Variables	Coded level of variables		
	-1	0	+1
Drug to solvent ratio (g/50 mL)	2.5	6.25	10
Temperature (°C)	30	45	60
Time (min)	15.5	27	40

**Table 3: Box-Behnken experimental runs**

Run	Factor-1-temperature (°C)	Factor-2-time (min)	Factor-3-drug to solvent ratio (g/50 mL)
1	30	27.5	10
2	60	27.5	2.5
3	60	27.5	10
4	45	27.5	6.25
5	60	40	6.25
6	45	27.5	6.25
7	30	15	6.25
8	45	40	2.5
9	45	27.5	6.25
10	30	27.5	2.5
11	45	40	10
12	45	15	2.5
13	45	27.5	6.25
14	45	27.5	6.25
15	30	40	6.25
16	60	15	6.25
17	45	15	10

### Quantification of embelin in *Embelia ribes* extracts

Quantification was done by high performance liquid chromatography (HPLC) conducted on SPD-10A VP (SHIMADZU, Kyoto, Japan) HPLC system comprising of Binary LC-10AT VP pumps, a single wavelength of UV-visible detector (programmable) and a system controller. Samples were injected using a rheodyne injector fitted with a 20 µL fixed loop. Standard and the samples were filtered by 0.45 µm syringe filter before injection in HPLC system. Separation was achieved through C-18 reverse phase column (Merck, Germany) with dimensions of 25 × 4.6 mm and particle size 5 µm. Methanol: water (88:12 v/v) and 0.1% aqueous solution of trifluoroacetic acid (50:50 v/v) in isocratic mode with a flow rate of 1 mL/min were used as mobile phase. The wavelength used for detection was 288 nm.

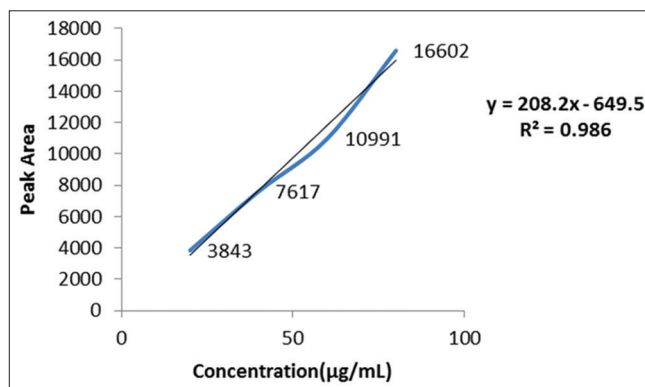
Calibration curve was prepared for peak area versus concentration for standard embelin [Figure 2]. Content of embelin in different extracts was then calculated from the calibration curve. Standard embelin was purchased from INDOFINE (Hills borough, NJ, USA).

## RESULTS AND DISCUSSION

### Optimization of extraction parameters for embelin

Content of embelin in extracts of *E. ribes* was determined by HPLC. Maximum embelin content was found in ethyl acetate extract viz. 23.71%. The content of embelin in different extracts of *E. ribes* is given in Table 4.

Single factorial experiments conducted showed effect of a particular parameter on embelin content when other two parameters were kept constant. Below are given the graphs showing the results of single factorial experiments. The conditions found to be the best



**Figure 2:** Calibration curve of standard embelin with respect to peak area

Table 4: Embelin content in different extracts		
Solvent	Extractive weight (mg)	Embelin content (%)
Methanol	353.03	19.80
Chloroform	300.07	16.27
Acetone	278.80	22.80
Ethyl acetate	345.00	23.71
Hexane	169.40	15.36
Diethyl ether	254.03	13.63

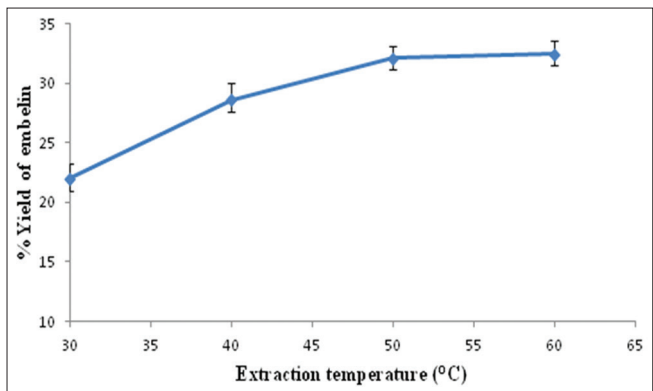


Figure 3: Effect of extraction temperature on embelin yield

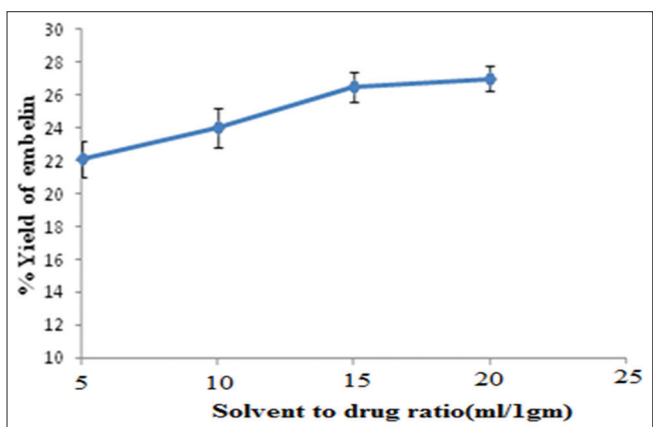


Figure 5: Effect of solvent: drug ratio on embelin yield

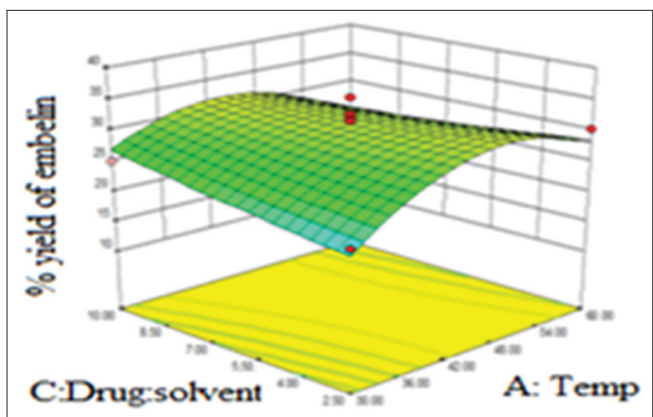


Figure 7: Dimensional response surface plot for embelin extraction showing interactive effects of temperature and drug: solvent ratio

for extraction of maximum embelin content were temperature (45°C), time (27.5 min) and solvent to drug ratio (6.25 mL/g) [Figures 3-5].

Depending on the results of single factorial experiments, BBD was employed for optimization purpose [Tables 2 and 3]. By using regression analysis on data of

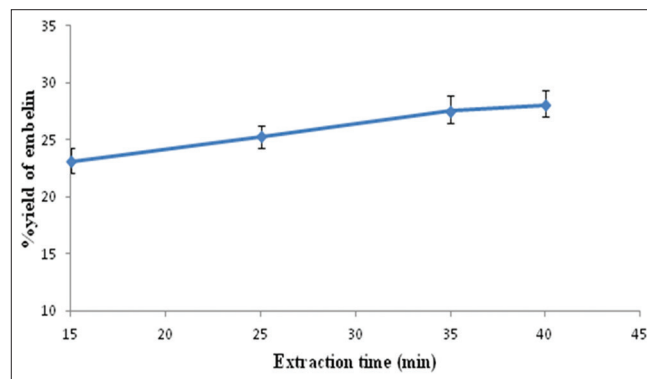


Figure 4: Effect of extraction time on embelin yield

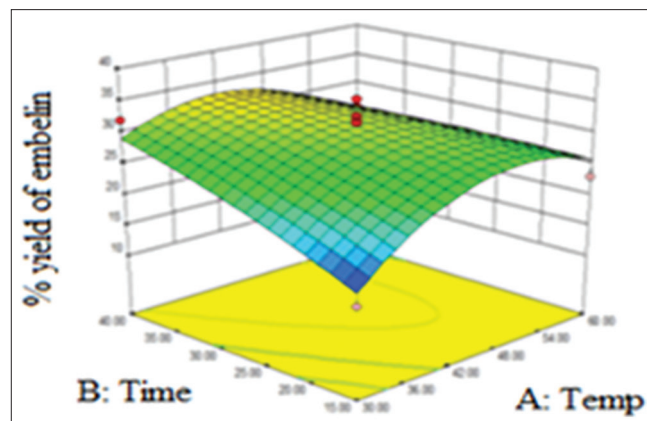


Figure 6: Dimensional response surface plot for embelin extraction showing interactive effects of temperature and time

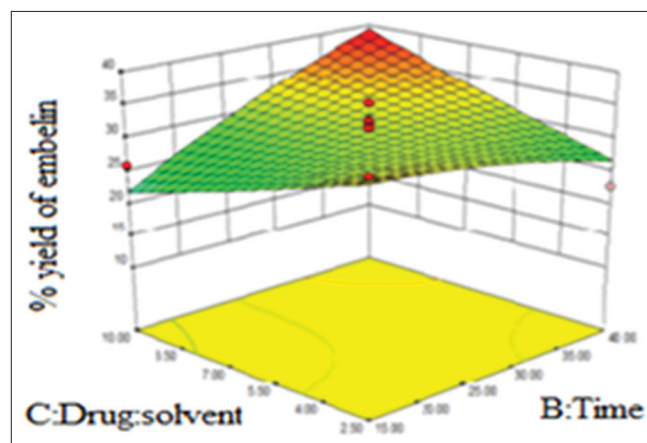


Figure 8: Dimensional response surface plot for embelin extraction showing interactive effects of time and drug: solvent ratio

experiment, the response and test variables were associated by the following quadratic equation:

$$Y = 31.07 + 1.10*A + 2.73*B + 0.421*C - 3.74*A*B - 2.62*A*C + 5.92*B*C - 6.34*A^2 - 1.009*B^2 + 0.302*C^2$$

Where,

A is extraction temperature (°C), B is extraction time (min) and C is a drug to solvent ratio (g/50 mL solvent).

The regression coefficient values, *P* values were used to determine the significance of each coefficient, which in turn demonstrates the pattern of interactions between the variables. In the study, the signal (response) to noise (deviation) ratio was found to be 6.752, which indicated a sufficient signal and, therefore, the model was important in extracting process. The value of  $RA_{adj}^2$  (0.4715) was nearly close to 1, indicating a degree of correlation between the observed and predicted values, hence suggesting that the model was significant. Analysis of variance for quadratic model is given in Table 5.

The three-dimensional (3D) response surface plot of embelin extraction for every pair of extraction parameters by preserving another constant at its intermediate level are presented in figures below. The contour plots for the same have also been depicted below.

Figures 6-8 shows the interactive effect of different parameters for embelin yield. Figure 6 shows the effect of extraction temperature and time when drug: solvent ratio was kept constant. 3D graph showed that the yield increased by increasing extraction time as well as temperature. However, furthermore increment in temperature decreased the yield. When time was kept constant and temperature and drug: solvent ratio was varied [Figure 7], the yield of embelin was enhanced with decreasing drug: solvent ratio and increment in temperature. Figure 8 shows that when temperature was fixed at zero level, and drug: solvent ratio and time were varied, yield of embelin first increased and then decreased with increase in extraction time and drug: solvent ratio. The corresponding contour plots have also been depicted in Figures 9-11.

#### Validation of the model

The conditions of optimal extraction for the extraction of embelin from *E. ribes* fruits were obtained by fitting the experimental data in equation given in earlier section. The optimal conditions are as follows: Ultrasound extraction temperature - 45°C; extraction time - 27.50 min; drug: solvent ratio 1:8 g/mL. The actual and the predicted values for % age yield of

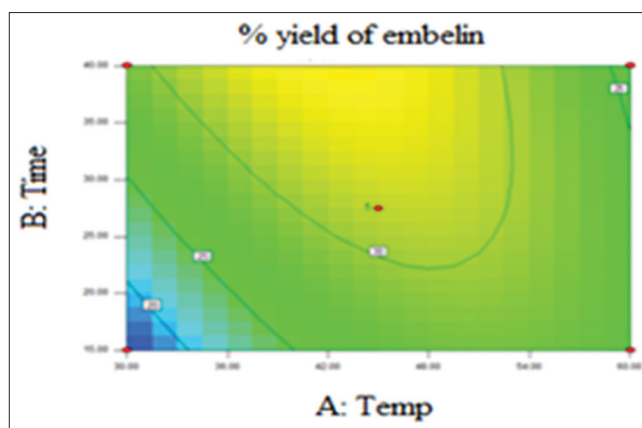


Figure 9: Contour Plot of temperature and time

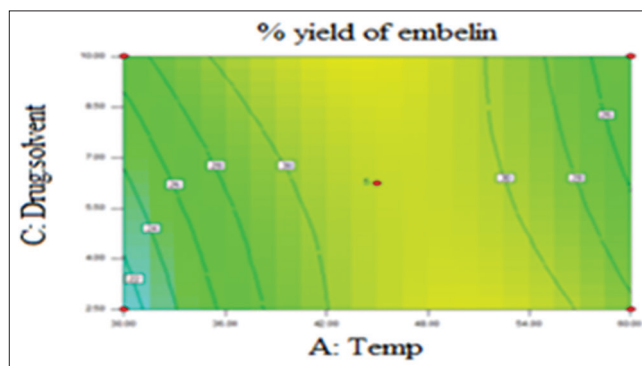


Figure 10: Contour plot of temperature and drug: Solvent ratio

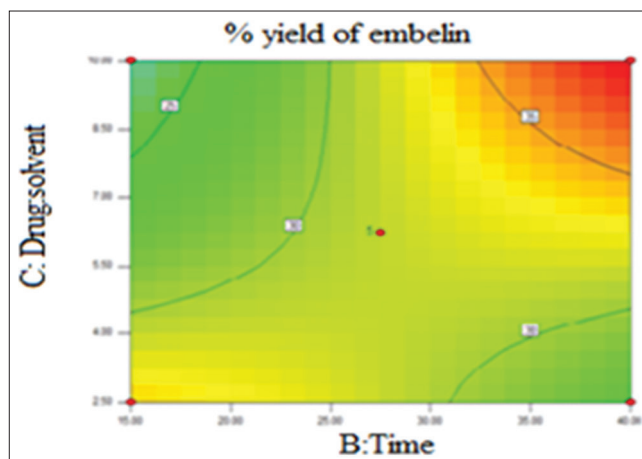


Figure 11: Contour Plot of time and drug: solvent ratio

embelin are enumerated in Table 6. At these optimal levels of extraction parameters embelin extracted from *E. ribes* fruits was 32.71% that was very near to the anticipated value of 31.07%.

#### Quantification of embelin in *Embelia ribes* fruits by high performance liquid chromatography

High performance liquid chromatography method with UV detector at room temperature was used for the

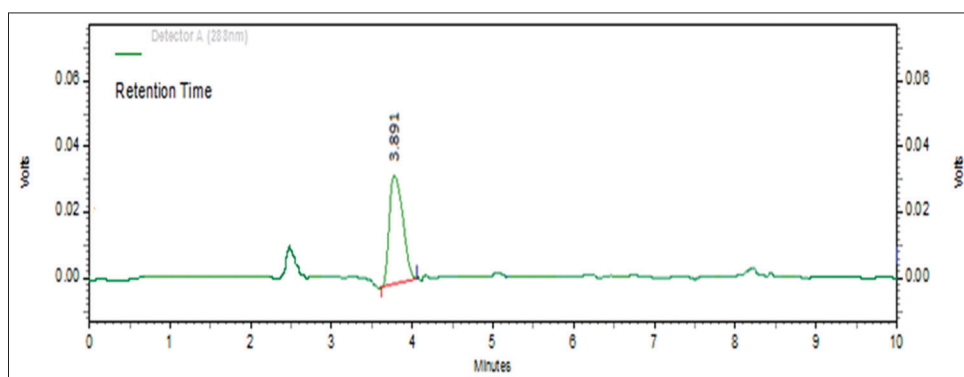
**Table 5: ANOVA for response surface quadratic model**

Source	Sum of square	Degree of freedom	Mean square	F	P Prob>F
Model	471.853	9	52.4317	2.586425	Significant
A-temperature (°C)	9.834613	1	9.834613	0.485136	0.5086
B-time (min)	59.95125	1	59.95125	2.95736	0.1292
C-drug: Solvent ratio	1.419613	1	1.419613	0.070029	0.7989
AB	55.9504	1	55.9504	2.760001	0.1406
AC	27.51003	1	27.51003	1.357054	0.2822
BC	140.1856	1	140.1856	6.915274	0.0339
A <sup>2</sup>	169.3648	1	169.3648	8.354666	0.0233
B <sup>2</sup>	4.293032	1	4.293032	0.211773	0.6593
C <sup>2</sup>	0.385927	1	0.385927	0.019038	0.8941
Lack of fit	66.75108	3	22.25036	1.184284	Not significant

**Table 6: Actual and predicted values for different experimental runs (BBD) for embelin extraction**

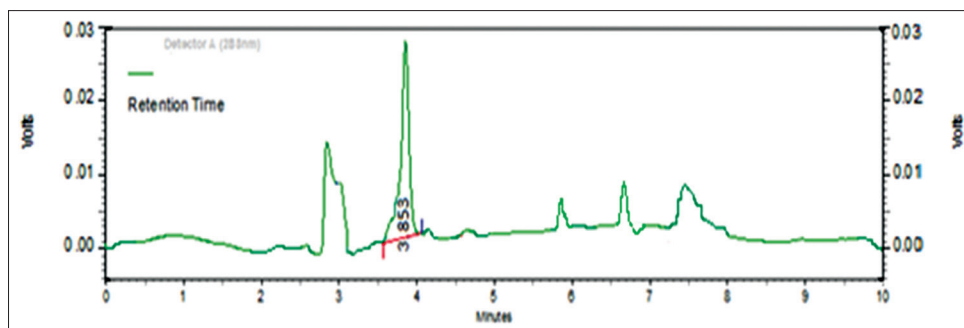
Run	Factor-1-temperature (°C)	Factor-2-time (min)	Factor-3-drug to solvent ratio (g/50 mL)	Actual values of % age yield of embelin	Predicted values of % age yield of embelin
1	30	27.5	10	25.006	26.96
2	60	27.5	2.5	30.25	28.34
3	60	27.5	10	22.86	23.94
4	45	27.5	6.25	32.71	31.07
5	60	40	6.25	25.85	23.26
6	45	27.5	6.25	32.07	31.07
7	30	15	6.25	14.11	16.13
8	45	40	2.5	22.83	26.76
9	45	27.5	6.25	31.56	31.07
10	30	27.5	2.5	21.96	20.88
11	45	40	10	38.5	39.44
12	45	15	2.5	34.07	33.12
13	45	27.5	6.25	35.27	31.07
14	45	27.5	6.25	23.25	31.07
15	30	40	6.25	31.94	29.08
16	60	15	6.25	22.98	25.83
17	45	15	10	35.27	31.07

BBD: Box-Behnken design

**Figure 12:** High performance liquid chromatography chromatogram of standard embelin (80µg/mL). Retention time - 3.891 min

determination of embelin in the fruits of *E. ribes* for 17 runs of BBD. Mobile phase consisted of methanol-water (88:12) with 0.1% trifluoroacetic acid in water and flow rate was 1 mL/min and elution was monitored at 288 nm. Total run

time was 10 min. The retention time of embelin in fruit extract was found to be 3.85 min [Figures 2, 12 and 13]. As shown in Table 6, maximum embelin content was found in the extract of run 11 that is, 38.5%.



**Figure 13:** High performance liquid chromatography chromatogram of *E. ribes* fruit extract. Retention time- 3.853 minutes

## CONCLUSION

Response surface methodology was adapted to reform the extraction parameters for extraction of embelin from the fruits of *E. ribes*. The parameters assessed included ultrasound extraction time, temperature and drug: solvent ratio. These were optimized employing BBD of RSM and the parameters of best extraction of embelin from the fruits of *E. ribes* was found to be ultrasound extraction temperature - 45°C; extraction time - 27.50 min; drug: solvent ratio - 1:8. Maximum embelin content under these conditions was found to be 32.71% which was nearly similar to the expected value of 31.07%.

The developed RP-HPLC analytical method may find an application in herbal drug industry for quantitative estimation and standardization of embelin from *E. ribes*.

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