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Research article

Extraction and identification of bioactive compound embelin from *embelia ribes* fruits

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ABSTRACT

Embelia ribes is the most widely used plant in numerous Ayurvedic preparations to treat various disorders. The most commonly used plant parts like leaves and fruit are in the Indian traditional system for CNS (Central Nervous System) disease, Anti-bacterial Anthelmintic, and Anti-ulcer activity. The proposed work aimed to optimize different extraction parameters (Part of Crude drug, Solvent and Temperature) to increase the extraction yield of the Embelin from *Embelia ribes* fruits. Extraction was done by using Chloroform as a solvent, and extraction yield was optimized by using independent variables, such as solvent, drug parts, time and temperature, with the help of the design of expert software. One part of the crude drug kept in 3 parts of Chloroform for 34 mins at 49 ºC temperature gives the maximum yield (38.73±1.13%) of Embelin. HPTLC was done by using the mobile phase of Chloroform: Ethyl acetate: Formic acid (5:4:0.5 v/v/v). A single band appeared in the TLC plate exhibiting the Isolation of a Single compound, further the Isolated compound was identified and characterized by using Ultraviolet Spectroscopy (UV), Fourier Transform Infra-Red (FTIR), and Nuclear Magnetic Resonance Spectroscopy (NMR). The results obtained from the analytical techniques reveal that the Isolated compound was Embelin.

Keywords: Embelin, Characterization, Extraction, HPTLC, NMR Spectroscopy.

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INTRODUCTION

Embelin is a chief phytoconstituent present in leaf [1] and fruiting parts of the *Embelia ribes* Burm F. It is a climbing herb belonging to the family Myrsinacae [2]. The name also knows as false black pepper or Vidanga [3]. And commonly found in deciduous forests distributed from the outer Himalayas to the Western Ghats [4]. Embelin is a benzoquinone compound; chemically, it is 2, 5- dihydroxy-3-undecyl-2, 5-cyclohexadiene-1, 4 benzoquinone. Moreover, widely used in the Indian medicinal system and Ayurveda. In Ayurveda, this plant is named as Vidanga or Bashmak. As per the Ayurvedic medicinal system, this plant has numerous clinical applications like Anthelmintic, Laxative, Carminative, etc., [5] and also adjuvant in many of the Ayurvedic preparation.

Many experimental works reported that the isolated Embelin compound has tremendous clinical potential activities like Wound healing activity [6], Anti-convulsant activity [7], Anxiolytic activity [8], Anti-ulcer activity [9], Anti-depressant activity [10], Anti-bacterial activity [11], Anti-mitotic activity [12] and Antipsychotic activity [13]. Different pharmacological activity and clinical significance indicate that Embelin is a potent phytoconstituent, used in various life-threatening diseases.

As a result, several efforts were made to extract, isolate, and identify Embelin from the plant extract. Embelin is chosen as a marker compound for the standardization of various Ayurvedic preparation consisting of *Embelia ribes* plant parts, due to its rich availability and therapeutic potency.

The course powder of *Embelia ribes* fruits was soaked in different polarities of solvents such as Methanol, Chloroform, Toluene, Ethyl acetate and Hexane at room temperature. However, the crystals (yield 10%) were observed only in the Chloroform fraction. The prime objective of the proposed work is to identify and maximize the output of crystals by varying the various parameters such as part of solvent, time and temperature.

MATERIALS AND METHODS

Materials

The Fruits were collected from the Palakkad district and authenticated by the Department of Quality Control Lab (Botany division), National Ayurveda Research Institute for Panchakarma, Kerala-679531. Silica gel 60 F_{254} TLC plate, HPLC grade of Chloroform, Ethyl acetate, Formic acid and Methanol are purchased from Merck (Germany). Embelin Standard was procured from Natural Remedies, Bangalore (Purity> 95% by HPLC).

Pharmacognostic evaluation

The Pharmacognostic characteristics of collected fruits

(*Embelia ribes*) have been analyzed. Morphological analyses were carried out according to the previous research methods [14]. The dried fruit of a plant is used for Micro-morphological analysis using a Trinocular stereo microscope (Unilab, India). A Transverse section of fruit was examined by a Compound microscope (Olympus, India). Pulverized dried fruits of *Embelia ribes* were used for Powder microscopy examination.

Optimization of extraction parameters of Embelin

Studying the effect of an independent variable (Drug solvent ratio, Extraction time, and Extraction temperature) on the Response (% Yield) was carried out by Randomized Response Surface Methodology. The lower and upper limit of each independent variable in terms of coded and un-coded Value was given in (Table 1).

Table 1: Coded and actual levels of Three Variables

Independent variable	Symbol	Coded level		
A: Part of solvent for 1 part of plant powder (Solvent: Powder)		-1	0	+1
	X1	1	2	3
B: Time (min)	X2	15	27.5	40
C: Temperature (°C)	X3	30	55	80

The experiment was performed using Design expert software, state-Ease version 13. Box-Behnken designs were selected, consisting of 17 experimental runs (Table 2).

The experiment was conducted randomly to predict the maximum yield in a limited experiment run. The following equation (01) exhibited the relationship between independent and dependent variables.

The Polynomial equation developed by BBD design is as follows**.**

Table 2: Box-Behnken Experimental Runs

Run	Factor 1 (A: Part of solvent for 1 part of plant powder)	Factor 2 (B: Time)	Factor 3 (C: Temperature)	Response 1 Yield (%)
	Part	Minutes	°C	%
1	2	40	80	33
2	2	40	30	34
3	1	27.5	30	23
4	1	40	55	29
5	2	27.5	55	37
6	3	27.5	30	31
7	2	15	30	30
8	3	27.5	80	33
9	2	27.5	55	37
10	3	40	55	39
11	1	27.5	80	22
12	2	27.5	55	37
13	1	15	55	25
14	2	15	80	29
15	2	27.5	55	37
16	2	27.5	55	37
17	3	15	55	36

The second order of the quadratic equation was used to predict the maximum values of Y (yield) by changing the Value of independent variables.

$$Y = b0 + b1A + b2B + b3C + b12AB + b13BC + b23BC + b11A2 + b22B2 + b33C2 - - - -(01)$$

Here Y represents response related to different factors like b0 is constant, linear coefficients (b1, b2, and b3), and interaction coefficients b12, b13, and b23. However, b11, b22, and b33 were the quadratic coefficients produced by variable experimental runs. At the same time, A, B, and C abbreviations were coded values assigned to the respective independent variable drug-to-solvent ratio, temperature (°C), and time (min), respectively.

Method of Isolation/Extraction

The extraction method was selected [15] and performed based on the experimental design's optimized formula. Accurately weigh one part of the dried coarse powder of *Embelia ribes* fruits. Kept in different proportions of Chloroform Solvent, an interval of Time and Temperature is given in Table 01. The obtained extract was filtered, and further, it was dried under reduced pressure by using a rotary evaporator (Model: IKA, RV 10 D). A small quantity of Chloroform was added to the extract and heated for a few minutes to get the clear solution and stored in a refrigerator overnight. Observed the bright orange colour, Embelin crystals. The obtained yield was correlated with the predicted yield to validate the model used in the experiment design.

Identification of the extracted compound by TLC and HPTLC

Apply 5, 7 and 9 µL of the test solution (*Embelia ribes* fruits Chloroform extract dissolved in Methanol), 3 µL Embelin standard solution (10 mg of Embelin dissolved in 10 mL of a standard flask and makeup with Methanol) and 5 µL obtained Crystals sample (1 mg crystal was dissolved in 1 mL of Methanol) on different tracks of a pre-coated silica gel 60 F_{254} TLC plate (E. Merck). The plate was developed in the solvent system Chloroform: Ethyl acetate: Formic acid (5:4:0.5 v/v/v). The developed plate was observed @ 254, 366, 540 nm and White light. For the Derivatization of the TLC plate, the p-Anisaldehyde sulphuric acid reagent was used, and the plate was heated to 105 °C for 10 minutes. Camag TLC visualizer captured an HPTLC chromatogram of extracted crystal and plant extract under different wavelengths @ 254, 366 and 540 nm. The plate was scanned (Camag, TLC Scanner IV, India) at above mentioned wavelengths. Detection and data evaluation studies were performed by Vision cats Software.

Identification of extracted crystal by UV-Visible Spectrophotometer and FTIR

Extracted 1 mg crystals was dissolved in Chloroform and made the volume up to 10 mL. Spectrum recorded using a spectrophotometer (Shimadzu 1800, Japan) at 250-400 nm. The maximum absorbance wavelength of the isolated compound was

recorded. Further Identification of functional groups was carried out by FTIR (Agilent Tech. CARY 630). A small quantity of extracted crystal was kept on the sample holder, and the spectra were in the range of 4000-600 cm^{-1} to determine the intensity of % Transmittance. Characteristics frequencies of the sample were interpreted for the Identification of the compound. All the experiment was carried out in triplicate.

Structure elucidation of extracted crystal by NMR Spectroscopy (^{1}H, ^{13}C, DEPT-90 & 135)

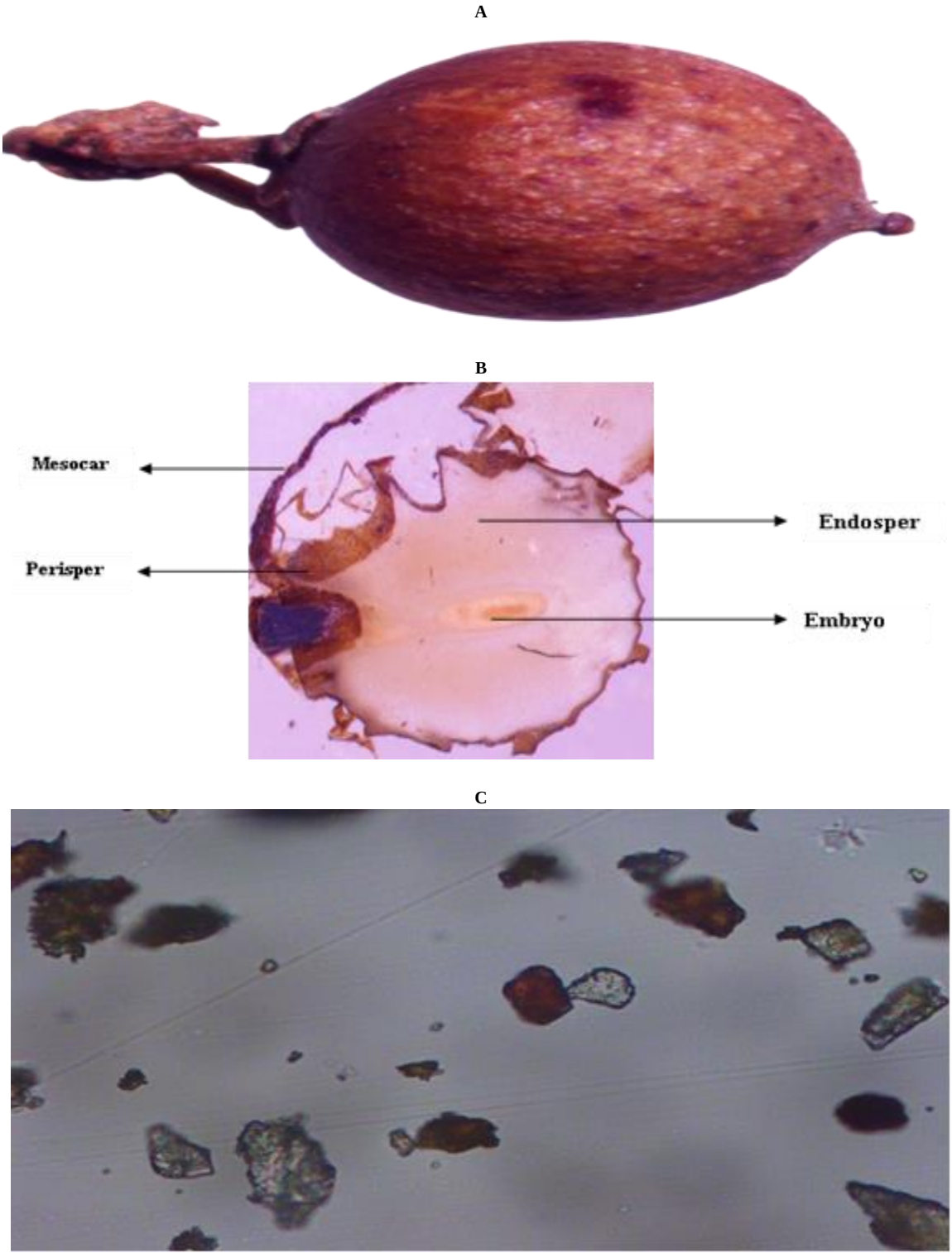
The NMR (model: FT-NMR Bruker Avance NEO 400 MHz) analysis of obtained crystal was performed at the Indian Institute of Science Education and Research (IISER) Tirupati. In the sample preparation, a selected solvent is $CDCl_3$ [16].

RESULTS AND DISCUSSION

Pharmacognostic evaluation

Macroscopic examination depicted that dried fruit *Embelia ribes* is blackish-brown coloured fruit obovate to sub globular shape,

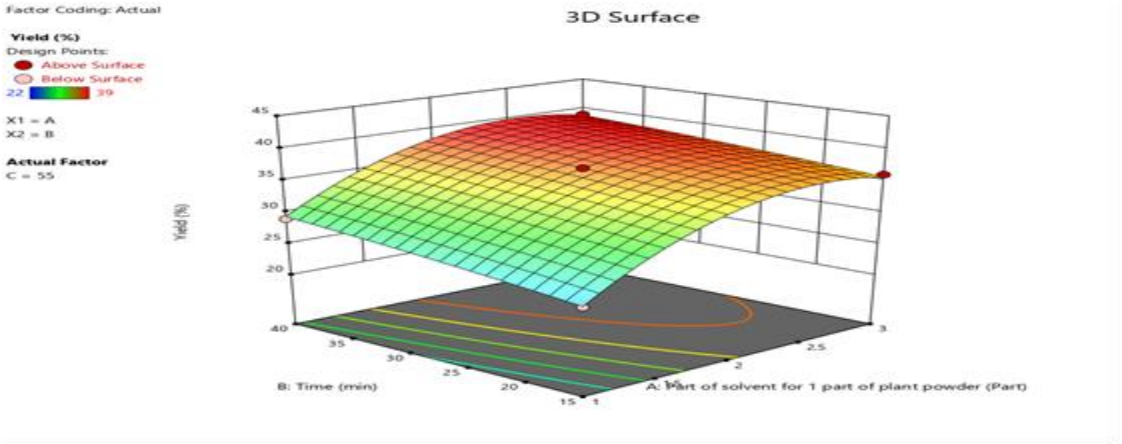
Figure 1: Pharmacognostic evaluation of *Embelia ribes* fruit (A); Macroscopic, (B): Microscopy, (C); Powder Microscopy



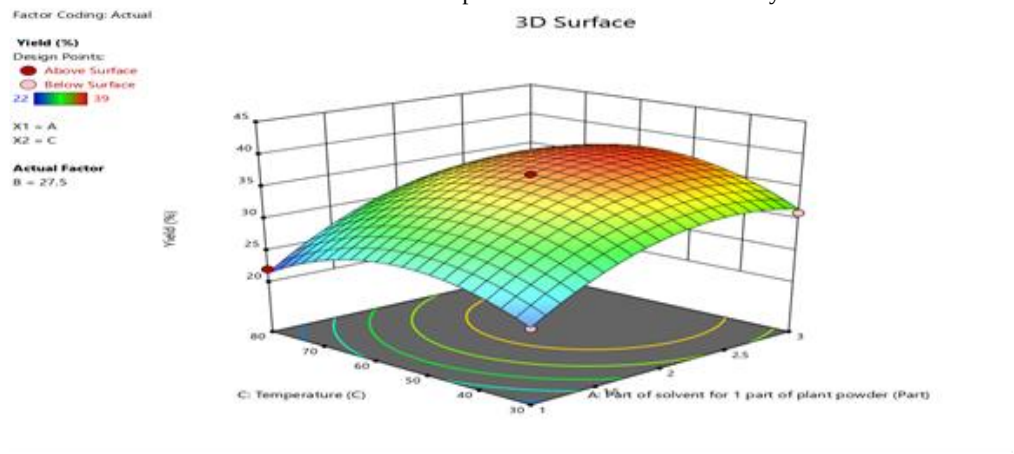
2-4mm in diameter. Beak-like projection at the apex, brownish-black seed with longitudinal furrows given in (Figure 1A).

Figure 2: Effect of different independent variables; A: Parts of solvent for 1 part of plant powder, B: Time (min's), C: Temperature (°C) on % Extraction Yield of Embelin

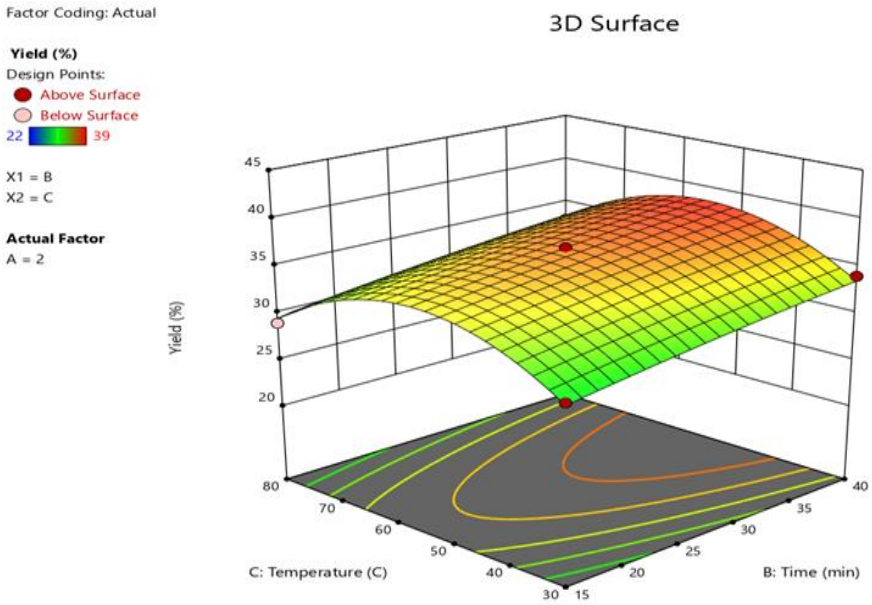
I: Effect of independent variable A and B on % yield of Embelin



II: Effect of independent variable A and C on % yield of Embelin



III: Effect of independent variable B and C on % yield of Embelin



Microscopic study of dry fruit exhibited the distinct grooved and ridged outline, mesocarp encircled by a layer of epicarp, is narrow, traversed by groups of stone cells and vascular strands. Underneath this lies a layer of dark brown inward intrusions of the perisperm inside the white endosperm, the embryo small, lying at the centre (Figure 1B). Powder microscopy shows abundant pitted lignified stone cells and sclereids of various sizes and thicknesses. Groups of abundant fragments of dark brown-coloured palisade-like cells of endocarp, fragments of perisperm, and endosperm filled with aleurone layer [18] are given in (Figure 1C).

Macroscopic and microscopic study of dried fruit and powder complies with the characteristic feature of *Embelia ribes* fruit given in API P-I Vol-I. It is an inference that the collected drug has good quality standard are free from any kind of physical, chemical, and microbial deterioration.

Effects of different parameters on Extraction

The Response Surface Method (RSM) exhibited the effect of different levels of the independent variable on the response (% yield) of Embelin (Figure 2).

The optimized condition found best for the maximum yield is given in (Table 6). Regression analysis was performed on the experimental data, by using the following equation

$$Yield=37+5A+1.88B-0.1250C-0.25AB+0.75AC+0.0BC-4.50A^2-0.25B^2-5.25C^2 \text{--------(02)}$$

A is part of the solvent for 1 part of the drug, B is extraction time (min), and C is Extraction temperature (°C). P-value (Regression coefficient) was employed to predict the significance of each coefficient (Table 4). The experimental signal-to-noise ratio was 45.634, exhibiting that the signal value of the model was significant, and the regression coefficient was 0.99, as given in (Table 4), which depicts the characteristic signal. Therefore, this model was best fitted and significant for the extraction process. 3D surface model graphs of extraction were made to analyze the % yield of Embelin at a different level of each independent variable by making other constants. (Figure 2) depicted that on increasing the part of the solvent, extraction efficiency increases. The % yield obtained at the intermediate and higher part of the solvent was nearly similar. It exhibited that the intermediate level of solvent is the appropriate amount of solvent required for the maximum extraction of Embelin from the 1 parts of the raw drug. The relationship graph between time and % yield describes the extraction of Embelin increases over time, and in the case of temperature, up to moderate temperature extraction increases. After that increases, the temperature extraction efficiency decreases due to the changes in the polarity of the respective solvent at a higher temperature. An appropriate value of each independent variable for the extraction of Embelin was analyzed by fitting the experimental value in equation (02).The ANOVA for Response Surface Quadratic Model (Table 3). Actual and predicted values of the independent variable and response are given in (Tables 5 and 6). The minimal differences, in the predicted and actual values, indicate that the proposed model was validated for maximum extraction of Embelin from *Embelia ribes* fruits.

Table 3: ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	445.78	9	49.53	198.12	< 0.0001
A-Part of solvent for 1 part of plant powder	200.00	1	200.00	800.00	< 0.0001
B-Time	28.13	1	28.13	112.50	< 0.0001
C-Temperature	0.1250	1	0.1250	0.5000	0.5024
AB	0.2500	1	0.2500	1.0000	0.3506
AC	2.25	1	2.25	9.00	0.0199
BC	0.0000	1	0.0000	0.0000	1.0000
A^2	85.26	1	85.26	341.05	< 0.0001
B^2	0.2632	1	0.2632	1.05	0.3390
C^2	116.05	1	116.05	464.21	< 0.0001
Residual	1.75	7	0.2500		
Lack of Fit	1.75	3	0.5833		
Pure Error	0.0000	4	0.0000		
Cor Total	447.53	16			

Table 4: Regression Coefficient

Regression Coefficient	Value
R^2	0.9961
Adjusted R^2	0.9911
Predicted R^2	0.9374
Adeq Precision	45.6344

Table 5: Actual and predicted values for different experimental runs (BBD) for Embelin extraction.

Run Order	Actual Value (% Yield)	Predicted Value (% Yield)
1	33.00	33.25
2	34.00	33.50
3	23.00	23.12
4	29.00	29.37
5	37.00	37.00
6	31.00	31.63
7	30.00	29.75
8	33.00	32.88
9	37.00	37.00
10	39.00	38.88
11	22.00	21.37
12	37.00	37.00
13	25.00	25.12
14	29.00	29.50
15	37.00	37.00
16	37.00	37.00
17	36.00	35.63

Table 6: Optimum Conditions, Experimental and Predicted values of response at optimized conditions.

Optimized condition	Coded levels	Actual levels
A	1.97	2.97
B	0.5024	33.78
C	-0.411	48.83
Response	Predicted levels	Actual levels
% Yield	37.9031	38.73±1.13

The extracted product was obtained in the form of a bright orange colour crystal, an inference that the observed condition was extracted pure compound. This method can act as a more economical

and simpler for the Isolation of a single compound without using multiple steps or any sophisticated separation techniques [19].

Identification of the extracted compound by TLC and HPTLC

HPTLC is one of the sophisticated techniques to identify the Phytochemicals from plant extracts [20,21]. Figure 3 (A, B, C & D) exhibited that obtained crystals have a single spot at R_f 0.42 which matches with Embelin Standard and extract has multiple spots in their chromatogram along with crystal; one spot appears at a similar R_f, portraying the presence of Embelin in the plant extract. AUC of 5 µL crystal (Figure 4) and extracted compound (Figure 5) were observed at White light, @254, 366 and 540 nm at a similar R_f value.

Figure 3: HPTLC profile of Embelin Standard {Left to Right; Track 1 (3µL)}, Embelin Crystal {Left to Right; Track 2 (5µL)} and *Embelia ribes* fruit chloroform extract {Left to Right; Tracks 3 (5µL), 4 (7µL) & 5 (9µL)}at different wavelength (A: @ White light, B: @254nm, C: @366nm, ad D: after derivitization @540nm)

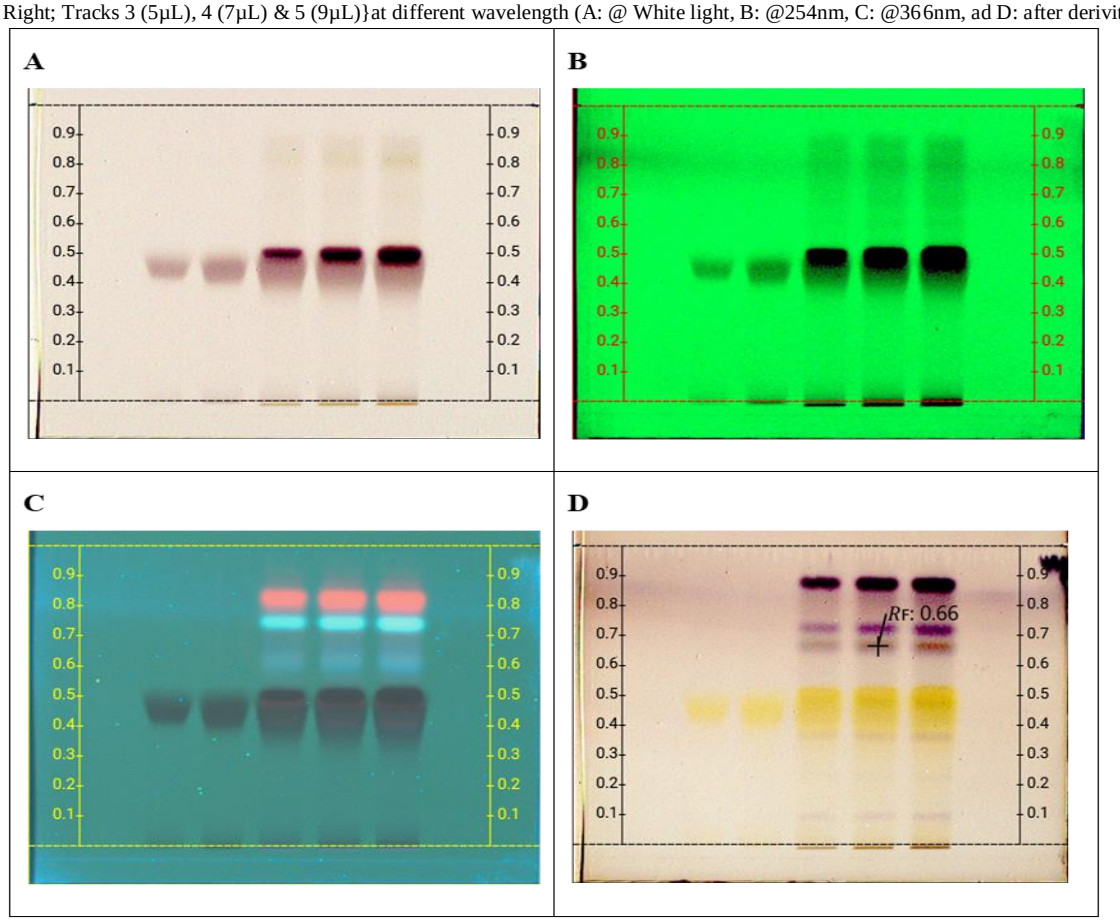
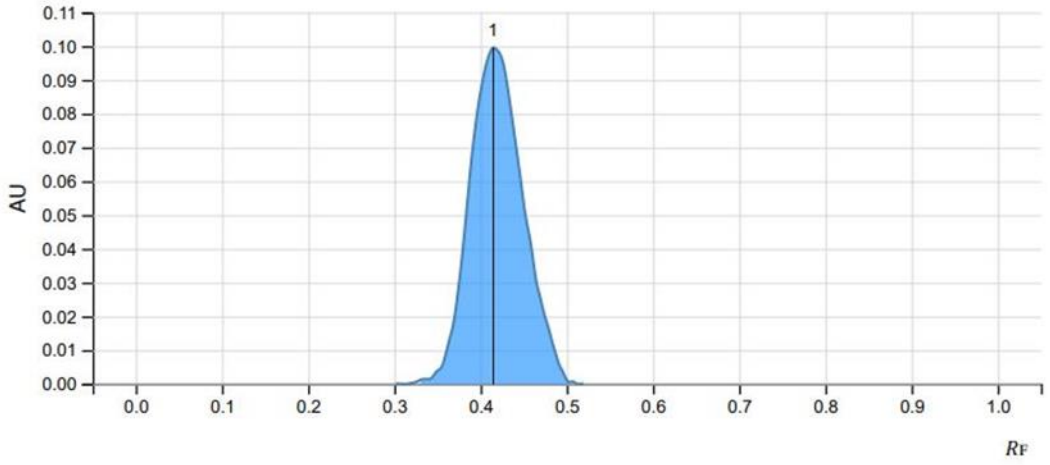
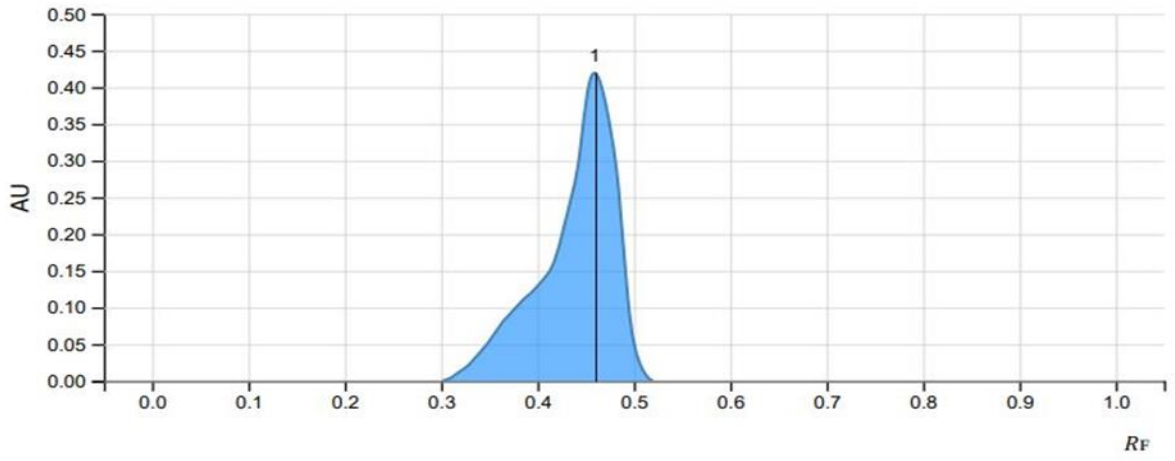


Figure 4: HPTLC chromatogram of Embelin crystal



Peak #	Start		Max			End		Area		Manual peak	Substance Name
	R_F	H	R_F	H	%	R_F	H	A	%		
1	0.331	0.0013	0.415	0.0995	100.00	0.503	0.0005	0.00729	100.00	No	embilin

Figure 5: HPTLC Chromatogram of *Embelia ribes* fruits chloroform extract



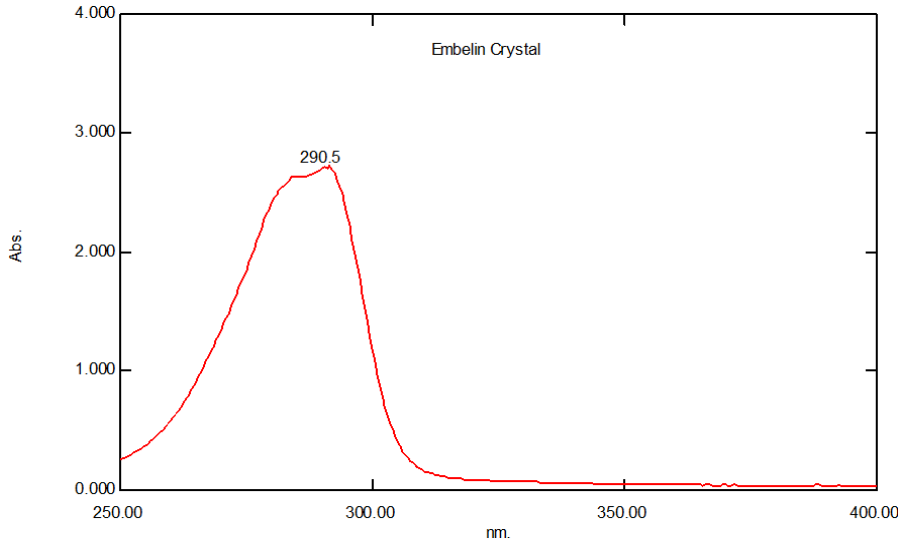
Peak #	Start		Max			End		Area		Manual peak	Substance Name
	R_F	H	R_F	H	%	R_F	H	A	%		
1	0.302	0.0000	0.460	0.4194	100.00	0.519	0.0002	0.03185	100.00	No	embilin

The appearance of the bright orange spot at similar R_f in both obtained crystals and plant extract depicted that Embelin can be used as a marker compound to characterize any Ayurvedic formulation preparation has *Embelia ribes* fruits.

A bright orange colour crystal was obtained. Zaid et al. [22] also reported morphology of Embelin was bright orange in colour crystal. % Yield of the Embelin was 38%. The melting point of the crystal was observed at 146 °C; similar melting points were reported by Nuthakki et al. [23]. This crystal gave a single spot at R_f 0.42 on TLC over silica gel 60 F_{254} (pre-coated aluminium plate, layer thickness 0.2 mm), using Chloroform: Ethyl acetate: Formic acid (5:4:0.5 v/v/v) as a mobile phase. It has a bright orange colour on dipping the plate in p-anisaldehyde-sulphuric acid reagent and heating at 105 °C to appear colour spots. Further, the compound was confirmed by UV, FTIR, and NMR Spectroscopy.

Identification of extracted crystal by UV-Visible Spectrophotometer

Figure 6: UV Spectrum of Embelin Crystal



UV Spectrum (Figure 6) exhibited that maximum absorbance was observed at 290.50 nm, a similar absorption wavelength reported by shrimali et al. [24]. Appearance single signal and maximum absorbance at specific wavelength inference that extracted crystal was Embelin.

Identification of extracted crystal by FT-IR Spectroscopy

The obtained crystal was characterized by FT-IR Spectroscopy, and Characteristic peaks were observed in the spectrum (Figure 7) are the Hydroxyl groups (3305 cm^{-1}), Methyl group (2921 cm^{-1}), and C-O (1194 cm^{-1}), Stretchings and Carbonyl group vibration (1618 cm^{-1}), values are matched with the previously reported of Kaur V [25]. It depicts that the resulting crystal was Embelin.

Figure 7: FT-IR Spectrum of Embelin Crystal

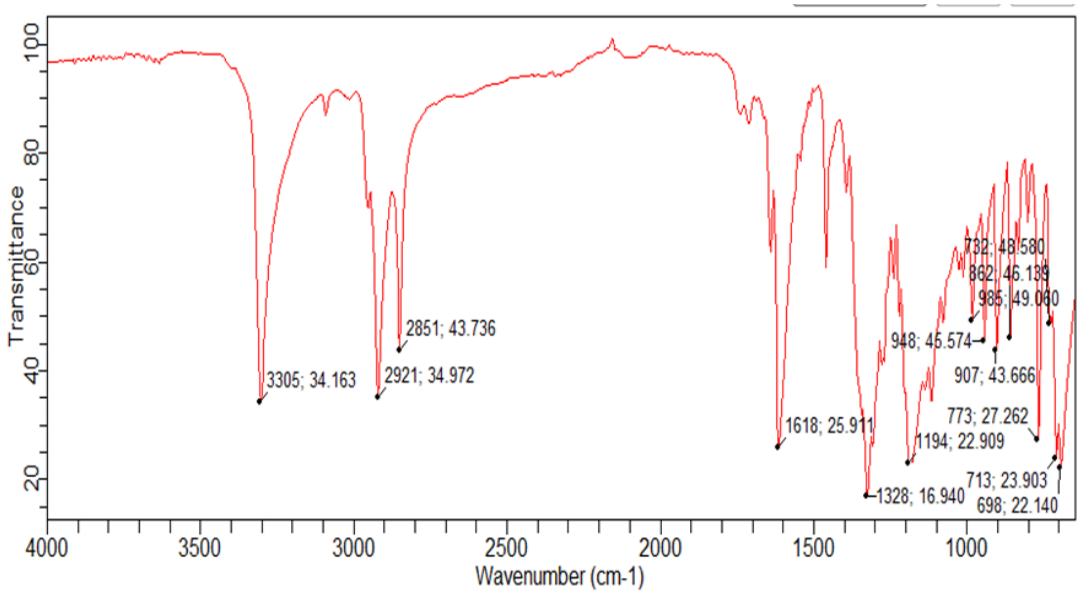


Figure 8: [1]H NMR Spectrum of Embelin Crystal

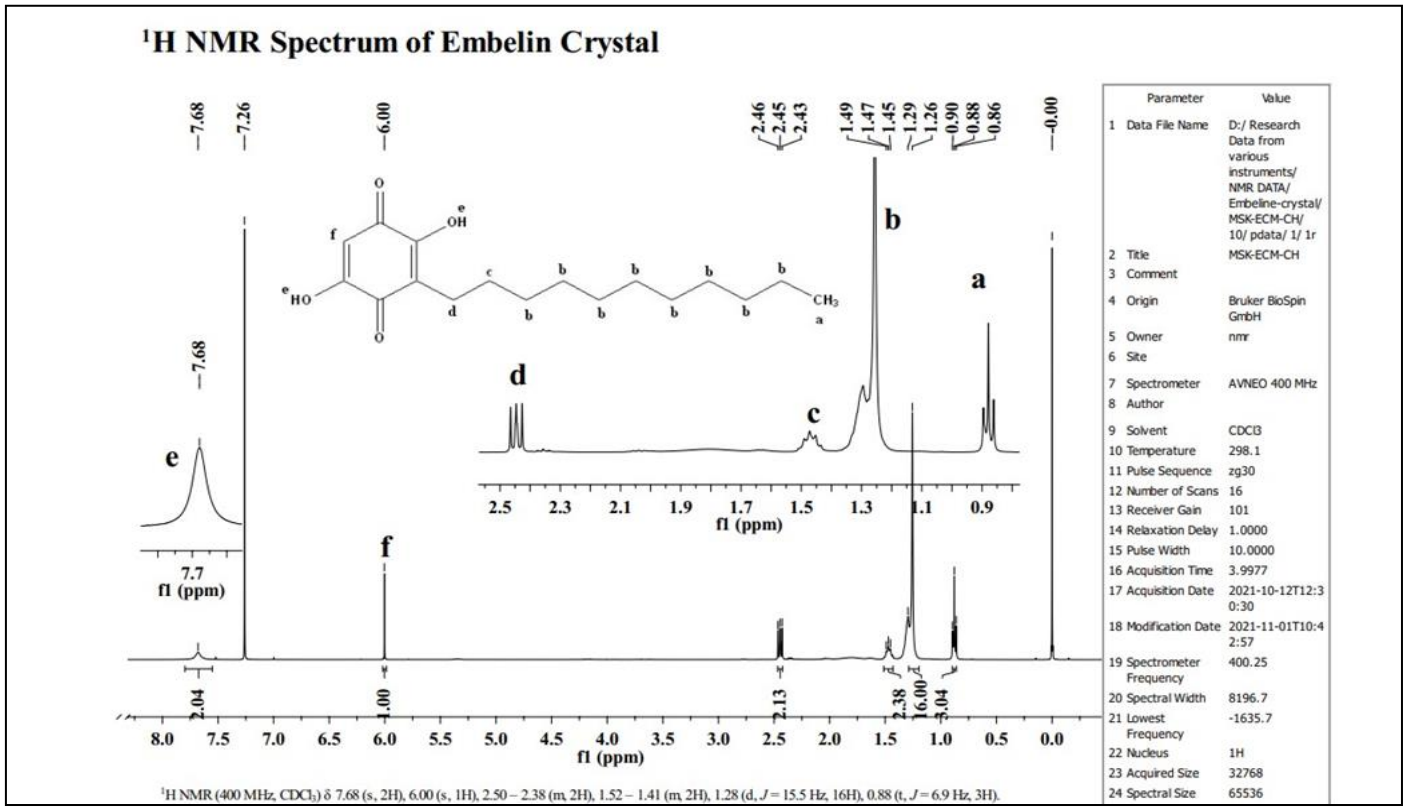
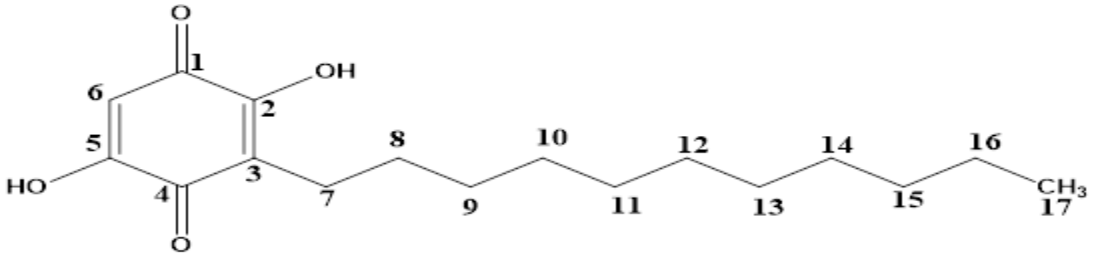


Figure 9: Chem. Draw the structure of Embelin



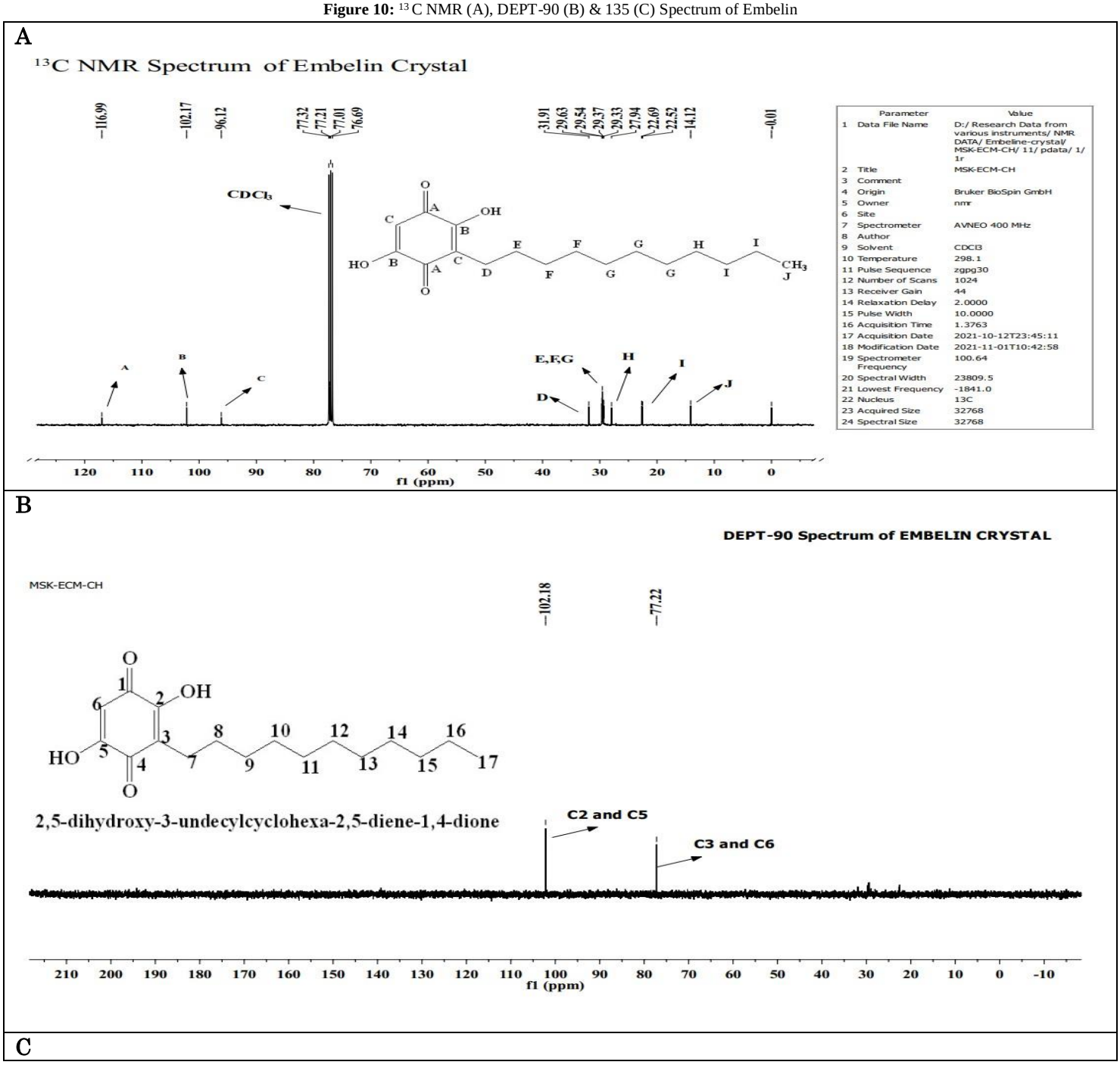
Structure elucidation of extracted crystal by [1]H, [13]C-NMR, DEPT-90 & 135

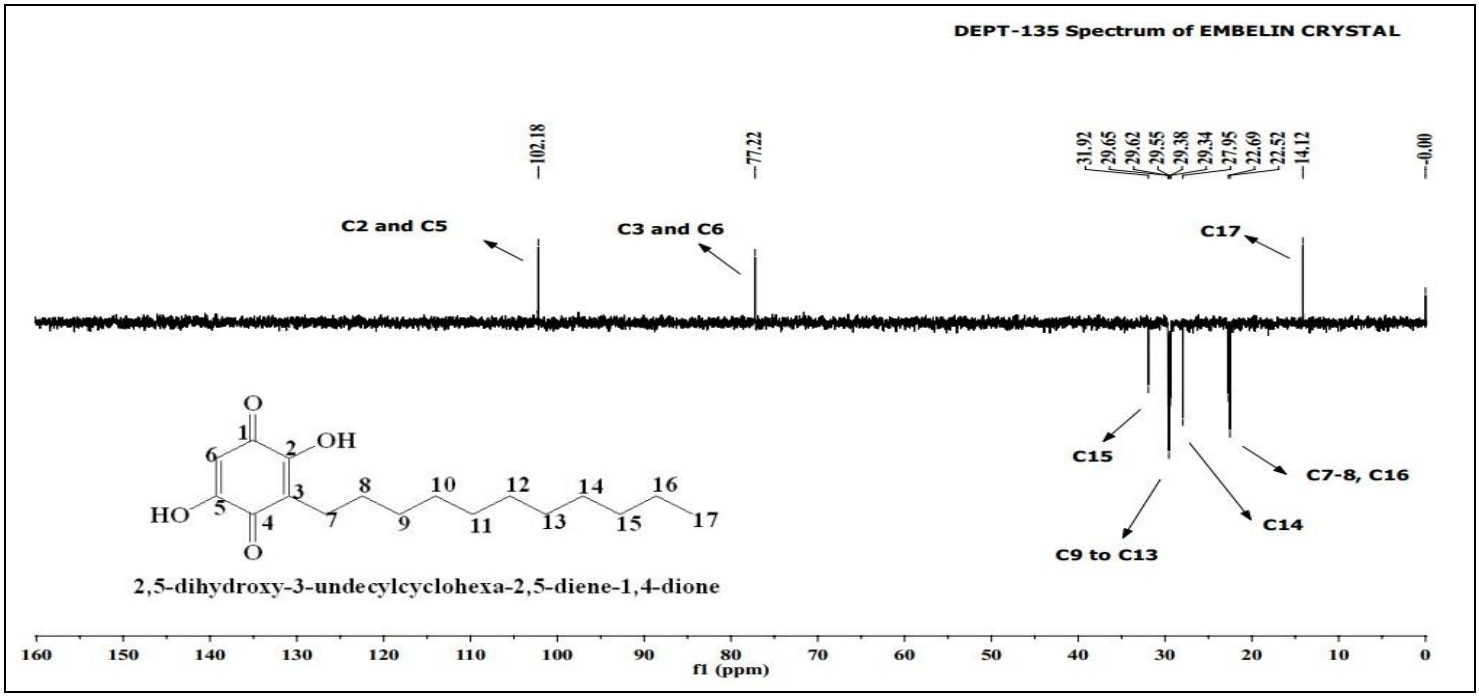
[1]H-NMR Spectrum (Figure 8) observed that 26

Characteristic hydrogens peaks at different chemical shifts are matched with the Chem. Draw structure of Embelin (Figure 9) and methyl hydrogens at C17 (a, 3H, triplet, δ 0.9), methylene hydrogens of C9-16 (b, 16H, multiplate, δ 1.3), methylene hydrogens at C8 (c, 2H, multiplate, δ 1.5), methylene hydrogens at C7 (d, 2H, triplate, δ 2.5), methyne hydrogen at C6 (f, 1H, singlet, δ 6.0), and hydroxyl hydrogens attached at C2, C5 carbon (e, 2H, singlet, δ 7.7).

The ^{13}C-NMR Spectrum (Figure 10A) Exhibits the Characteristic peaks of the Methyl group (J) at δ 14.12, Methylene groups (D, E, F, G, H, and I) at δ 22.52 to 31.91. However, the Carbonyl groups (A) at δ 116.99, Hydroxyl groups attached Carbons (B) at δ 102.17, and Alkene Carbons (B) at δ 96.12 reveal that extracted crystal was Embelin. Further structure elucidation was carried out by DEPT-90 & 135. The DEPT-90 Spectrum (Figure 10B) Exhibit Characteristic peaks of Tertiary carbons (C2 & C5) at δ 102.18 and (C3 & C6) at δ 77.22, respectively, and the DEPT-135 Spectrum (Figure 10C) exhibits the Primary and Tertiary carbons denote by positive sign and Methylene groups (CH_2) indicated by the negative sign. The Primary Carbon (C17) appears at δ 14.12, and Tertiary Carbons peaks appear (C2 & C5) at δ 102.18 and (C3 & C6) at δ 77.22, respectively. The Methylene group Carbons (C7 to C16) appear at δ 22.52 to 31.92. The obtained δ values of Embelin crystals were matched with the previously reported literature [26].

Figure 10: ^{13}C NMR (A), DEPT-90 (B) & 135 (C) Spectrum of Embelin





CONCLUSION

Based on the obtained results, it was concluded that the obtained isolated compound has a pure crystal of Embelin, structure elucidated by different analytical techniques confirmed that the extracted compound consists of Embelin and the 2.97 parts of the solvent, 34 min, and 49 °C temperature is a most suitable condition for the maximum extraction of Embelin crystal, around 38 % yield was easily obtained by this condition within the half-hour. This optimized condition can be used for faster and high extraction of Embelin. The high extractive value of Embelin also depicted that this compound can be used as a marker compound for the characterization of Ayurvedic Formulation consisting of plant parts of *Embelia ribes*.

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Conflict of interest:

The authors have no conflicts of interest regarding this investigation.

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