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Standardization Of Process Of Extraction Of Resveratrol From Underutilized Byproduct Jamun Seed (Syzygium Cumini) Using Ultrasonication

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Abstract
Resveratrol is a well-known antioxidant of considerable interest owing to its numerous beneficial health benefits. Jamun seed is an underutilized by-product and is a good source of resveratrol, offering a potential value-added product for these industries. The current investigation aimed to determine the best polyphenol extraction conditions of jamun seed using ultrasound assisted extraction procedure. Response surface methodology was applied to standardize the extraction variables such as process time, solvent concentration, temperature and sonication amplitude to maximize extraction yield. The optimized extraction conditions were 90 minutes, 85% ethanol, 55℃ and 50 Hz. Among the variables analysed for jamun seed extract, total phenolic content was estimated to be 315.92 mg GAE/g while antioxidant capacity as assessed by different assays, namely DPPH, ABTS and FRAP was recorded as 1326.68, 1803.27 & 1174.16 mmol TE/g. The qualitative analysis performed by high performance liquid chromatography demonstrated the presence of resveratrol in the extract.
Keywords: *Antioxidant, extraction, resveratrol, underutilized, variables*

INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene) is considered an important antioxidant belonging to stilbene class of polyphenols [1].The antioxidant is generally synthesized by plants subsequent to exposure to adverse condition like stress, injury, or fungal infection and hence is known as phytoalexin. It is a main bioactive component found in different common sources like peanuts, grapes, cocoa beans and red wine as well as underutilized ones like berries (blueberries, mulberries & Indian Blackberry) [2], [3]. Among these sources, Indian blackberry (Syzygium cumini) has been explored by a few scientists [4], [5], [6] while its byproducts also hold enormous potential to be investigated as a good source of the resveratrol. Jamun seed, a byproduct of this fruit, can contribute to agro-industrial pollution; therefore, its valorization as a potential source of the antioxidant provides a sustainable and environmental-friendly strategy of waste management as well as bioactive recovery [7].

Scientists have shown considerable interest in resveratrol due to its numerous beneficial health benefits including anticarcinogenic, anti-thrombotic, antimicrobial, antidiabetic anti-inflammatory and antioxidant capacities [8], [9], [10] . Besides this, it has gained prominence due to its predisposition to phenomenon called the "French Paradox" which refers to low occurrence of cardiovascular disease among French people despite consumption of high-fat diet (Drosu et al., 2015). Scientists have proposed red wine intake as a plausible explanation to it as it is consumed higher in France compared to Western countries. Epidemiological studies have shown that red wine contains a notable compound, resveratrol, responsible for inhibition of LDL oxidation. Oxidised lipids in phospholipid membrane have been associated with occurrence of lesions in arteries and hence, are responsible for atherogenic blockage in coronary heart disease (CHD) [11].

Based on abundant health benefits associated with resveratrol, a tremendous work has been in past years for optimization of isolation, extraction and purification of the compound. Conventional methods involve extraction by refluxing with solvent, soxhlet extraction, and maceration. In recent years, these methods have been replaced by latest techniques like microwave assisted extraction, Supercritical fluid extraction (SFE), enzymatic hydrolysis and Ultrasound assisted extraction (USAE). USAE has been demonstrated to be one of the latest simplest and rapid techniques for efficient extraction of bioactive components from complex matrix [12].

However, most of techniques are based on considering one-variable-at-a-time (OVAT) which is inefficient approach and does not take into account interactions between variables. Therefore, different variables involved in extraction process such as temperature, time and solid to solvent ratio require optimization to achieve the highest extraction efficiency.

Response surface methodology (RSM) is a popular statistical tool applied for optimization of extraction parameters since multiple variables and their interaction can be assessed simultaneously for developing mathematical models, which can establish the relation between response and independent variables. This methodology not only improves process efficiency but also assists in exploring optimal conditions for maximum resveratrol recovery with minimal resource input. The literature survey shows that there is a dearth of studies available for optimizing the ultrasound assisted extraction of resveratrol from a byproduct like jamun seed using RSM. Therefore, the current study was undertaken to model the USAE of resveratrol from jamun seed using linear regression using response surface methodology.

MATERIALS AND METHODS

Plant Materials

Jamun fruits were purchased from a local store in Delhi, India. The jamuns were washed and their seeds were removed manually. The seeds were dried at ambient temperature and ground using an electrical grinder following by passing through 0.5 mm screen to get a homogenous fine powder. The powder thus obtained were used for further extraction.

Preparation of jamun seed extract

To optimize the extraction conditions, one gram of jamun seed powder was extracted using 30 ml of extraction medium (ethanol) using a ultrasonicator (Hoverlabs Pvt. Ltd., India). After extraction, the samples were centrifuged (8000 g, 15 minutes at 4°C) and the solvent was removed using a rotary evaporator (R-205, Buchi, Switzerland). The extract thus obtained was kept in storage at -20°C until further analysis. The % yield was calculated from the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of extract}}{\text{weight of raw material}} \times 100 \qquad (1)$$

Optimization by response surface methodology (RSM)

In this study, the parameters used for extraction yield from raw material by ultrasound assisted extraction were analyzed by RSM technique employing a full factorial Central Composite Design with 30 experiments. Important independent variables (4) were selected for the statistical evaluation namely ethanol concentration (X_1, vol.%), time (X_2, min), temperature (X_3,°C), and sonication amplitude (X_4). The range and level of the variables changed in accordance with the experimental design. These four factors along with their corresponding ranges were observed to be crucial parameters for effective extraction yield from jamun seed. "Minitab 18" statistical software was employed to generate the response surface plots and to perform the regression analysis of experimental data. Analysis of Variance (ANOVA) was used for the estimation of statistical parameters. To ensure a good model fit, the regression model and individual model coefficients were tested for significance along with lack-of-fit test.

A quadratic polynomial model was used to fit the response variable as illustrated in the equation below:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{1,1}X_1^2 + \beta_{2,2}X_2^2 + \beta_{3,3}X_3^2 + \beta_{4,4}X_4^2 + \beta_{1,2}X_1X_2 + \beta_{1,3}X_1X_3 + \beta_{1,4}X_1X_4 + \beta_{2,3}X_2X_3 + \beta_{2,4}X_2X_4 + \beta_{3,4}X_3X_4 + \varepsilon \qquad (2)$$

Where, Y is the predicted response variable; β_0 is the constant regression coefficient; β_1, β_2, β_3, β_4 are the coefficients of linear effect; $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$, $\beta_{4,4}$ are the coefficients of quadratic effect; $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{1,4}$, $\beta_{2,3}$, $\beta_{2,4}$, $\beta_{3,4}$ are the coefficients of interaction effect. and ε is the experimental error.

Table 1. Different levels (coded) and values (experimental) for the independent variables applied in the CCD.

		Levels		
Factors	**Coded**	**-1**	**0**	**+1**
Ethanol concentration (%)	X_1	50	70	90
Time (minutes)	X_2	30	60	90
Temperature (°C)	X_3	40	55	70
Sonication amplitude (Hz)	X_4	40	50	60

Total phenolic content and Antioxidant capacity of jamun seed extract

TPC of jamun seed extract was estimated by using the Folin-Ciocalteu procedure proposed by Singleton and Rossi [13] with minor changes. A calibration curve using gallic acid standard was developed and the results were reported as mg of gallic acid equivalent per g of sample (mg GAE/g).

The free radical scavenging capacity of the sample was assessed using the DPPH assay procedure as given by Brand-Williams et al. [14], with some modifications. Each Sample extract was diluted 10 times, making the final volume up to 100 ml, subsequently adding 3.9 ml of DPPH solution (125 mM/L) prepared in

methanol. The prepared solution was incubated for half an hour under dark conditions at ambient temperature, and absorbance readings were taken at 517 nm with UV- Vis spectrophotometer (Ultrospec 1100 Pro, Biomicron Ltd., Cambridge, England). A calibration curve using Trolox standard with concentration varying in the range of 100-1000 mM/L methanol was developed and the results were reported as mmoL Trolox equivalent per g of sample (mMol TE/g).

The grape extracts were evaluated for their ABTS scavenging activity in triplicate following the protocol given by Re et al. [15], employing small changes. A solution was prepared by combining 10 ml each of ABTS (7 mMol/L) and potassium persulfate (2.45 mM/L) with distilled water as diluent. This was followed by mixing aliquots of 3 mL from each stock solution in a separate test tube and incubating the derived solution in the dark for 16 h at room temperature (25 ℃). Subsequently, the solution absorbance was adjusted to 0.70 at 734 nm by adding 250 mL distilled water to 4.0-4.5 mL ABTS radical. Jamun seed extract was diluted 100 times with distilled water in amber colour centrifuge tubes and ABTS solution (1700 mL) was added to it. Vortexing of the solution obtained thereof was done. This was kept undisturbed for 30 minutes maintaining darkness, followed by noting absorbance at 734 nm. The standard curve was plotted the using varying concentrations of aqueous Trolox (100-1000 mmol/L) and results were indicated as mmol TE/g of extract.

The ferric ion reducing antioxidant power (FRAP) assay was used to determine the total antioxidant potential of the grape extracts as per methodology of Benzie and Strain [16], with small modifications. An acetate buffer (300 mmol/L) with pH of 3.6 was mixed with 10 mM TPTZ in HCl (40 mM) and aqueous $FeCl_3$ (20 mM) in a ratio of 10:1:1 (v/v/v) for obtaining FRAP reagent. Also, jamun seed extract was diluted by taking 25 ml of the extract and making the volume up to 100ml. Subsequently, aliquot (3.0 mL) from the reagent as prepared above and 100 mL of diluted jamun seed extract were mixed briefly. The prepared mixture was kept in the dark for 30 minutes for incubation. Spectrophotometric determination of absorbance was done at 593nm. The standard curve was plotted at different concentrations of Trolox (100-1000 mmol/L) and observations were recorded as mmol TE/g of jamun seed extract. Triplicate analyses were done for all the tests.

HPLC Analysis

For data collection and processing, a Waters (Milford, MA) high performance liquid chromatographic system consisting of a fluorescence detector connected to the Waters Empower (version 5.0), a 717Plus autosampler, a 600-MS controller and a 996 photodiodearray detector (DAD) was employed. Separation was carried out at room temperature on a reversed phase Nova-Pak C_{18} column (250 × 4.6 mm r.d., 4 µm) (Waters, Milford, MA). At a flow rate of 1.0 mL/min, a gradient comprising solvents A (water/acetic acid, 98:2, v/v) and B (water/acetonitrile/acetic acid, 73:25:2, v/v/v) was applied as follows: Following 0–80% B linear from 0–55 minutes, 80–90% B linear from 55–57 minutes, 90% B isocratic from 57–70 minutes, 90–95% B linear from 70–80 minutes, and 95–100% B from 80–90 minutes, the column is washed (with methanol) and equilibrated from 90–120 minutes. Standard curve for resveratrol was developed using varying concentration of pure standard ranging from 5-50 ppm. An injection volume of 75 µL volume of extracted sample was inserted into the column of HPLC system and the peak for trans-resveratrol was detected using UV detector (at 303 nm).

Statistical analysis

Minitab (version 8.0.7.1, USA) was utilized to assess the optimum conditions of different input/ independent variables using three-dimensional response surface plots. The adequacy of the model was evaluated by ANOVA (analysis of variance), R^2 (determination coefficient) and adjusted R^2. SPSS statistical package (v. 16, SPSS IBM, USA). The mean differences were compared using one way ANOVA and the significant differences were reported for $p<0.05$.

RESULTS AND DISCUSSION

Optimization of extraction variables

Development of model using RSM

Table 2. Experimental design and experimental response variable data

Run	Ethanol conc. (%)	Time (min)	Temperature (°C)	Amplitude (Hz)	Yield (%)
1	70	60	55	40	38.56
2	70	90	55	50	41.76
3	50	60	55	50	39.89

4	70	60	70	50	43.78
5	70	60	40	50	42.89
6	70	30	55	50	39.12
7	70	60	55	50	40.99
8	70	60	55	60	42.23
9	70	60	55	50	40.67
10	90	60	55	50	41.33
11	70	60	55	50	40.45
12	90	30	40	40	35.12
13	50	30	40	60	36.99
14	90	90	70	60	44.67
15	90	90	40	60	43.56
16	50	90	70	60	42.99
17	90	90	40	40	36.15
18	50	30	70	40	37.22
19	90	30	70	60	40.89
20	50	30	70	60	38.12
21	50	30	40	40	34.06
22	70	60	55	50	40.09
23	70	60	55	50	40.26
24	90	90	70	40	41.98
25	50	90	40	40	35.56
26	90	30	40	60	36.63
27	70	60	55	50	40.99
28	50	90	40	60	41.67
29	50	90	70	40	37.79
30	90	30	70	40	39.04

The experimental data and observed responses of yield (%) of jamun seed extract using ultrasound extraction are given in Table 2. The analysis of variance of the linear, quadratic and interaction terms (Table 3) showed that the entire linear as well as the quadratic parameters were observed to be significant at the level of $p<0.05$. Whereas among the interaction parameters, X_1X_2, X_1X_4 and X_2X_3 were found to be insignificant ($p>0.05$). The output obtained by applying regression analysis on the experimental data was fitted into the second order polynomial mathematical equation and the final predictive equation (Eq. 3) was obtained by discounting the non-significant parameters. In order to fit a good model, analysis of Lack of Fit, R^2, adjusted R^2, F value and p value was performed.

Table 3. ANOVA for response surface model for % yield of jamun seed extract

Parameters	Sum of squares	Mean square	F value	P value	Significance
Model	212.496	14.1664	28.81	< 0.0001	Significant
X_1	12.634	12.6337	25.69	< 0.0001	Significant
X_2	46.529	46.5291	94.62	< 0.0001	Significant
X_3	31.601	31.6013	64.26	< 0.0001	Significant
X_4	57.853	57.8529	117.64	< 0.0001	Significant
X_1^2	2.284	2.2837	4.64	0.049	Significant
X_2^2	3.176	3.1756	6.46	0.024	Significant
X_3^2	8.011	8.0114	16.29	0.001	Significant
X_4^2	3.436	3.4363	6.99	0.019	Significant
X_1X_2	0.585	0.5852	1.19	0.294	
X_1X_3	3.312	3.3124	6.74	0.021	Significant
X_1X_4	0.176	0.1764	0.36	0.559	
X_2X_3	0.245	0.2450	0.50	0.492	
X_2X_4	12.638	12.6380	25.70	<0.0001	Significant
X_3X_4	3.349	3.3489	6.81	0.021	Significant
Lack of Fit	6.376	0.6376	5.02	0.067	

Pure error	0.508	0.1271	
R^2			0.9686
Adjusted R^2			0.935
Correlation total	219.381		

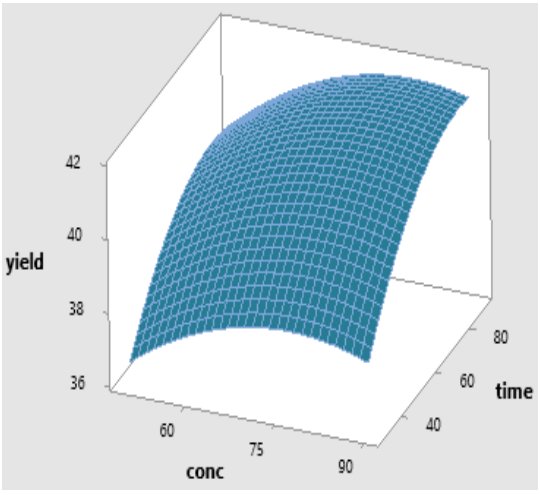
X_1: Ethanol concentration; X_2: Time (min); X_3: Temperature (℃); X_4: Amplitude

As shown in Table 3, a value of 0.9686 was obtained for R^2 which denoted a variation of 96.86% for ultrasound extraction of jamun seed. This was ascribed to the independent factors and the model could not explain only 3.14% of the variation. For a good statistical regression model, the value of adjusted R^2 should be comparable to R^2 (Dahmoune et al. 2015). The values of R^2 and adjusted R^2 did not differ considerably (Table 3). The statistics showed that the "Lack of Fit" F-value of 5.02 implies that Lack of Fit was insignificant verifying the validity of the statistical model. These results showed that the designed model could be reliable for the extraction of jamun seed.

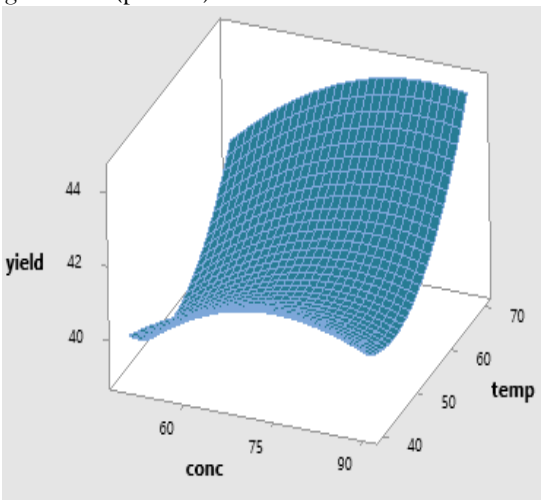
$$Y(\text{Yield}) = 4.6 + 0.298\,X_1 + 0.0474\,X_2 - 0.717X_3 + 1.370\,X_4 - 0.00237\,X_1^2 - 0.001243\,X_2^2 + 0.00790\,X_3^2 - 0.01163\,X_4^2 + 0.001517\,X_1X_3 + 0.002962\,X_2X_4 - 0.00305\,X_3X_4 \quad (3)$$

Analysis of response surfaces

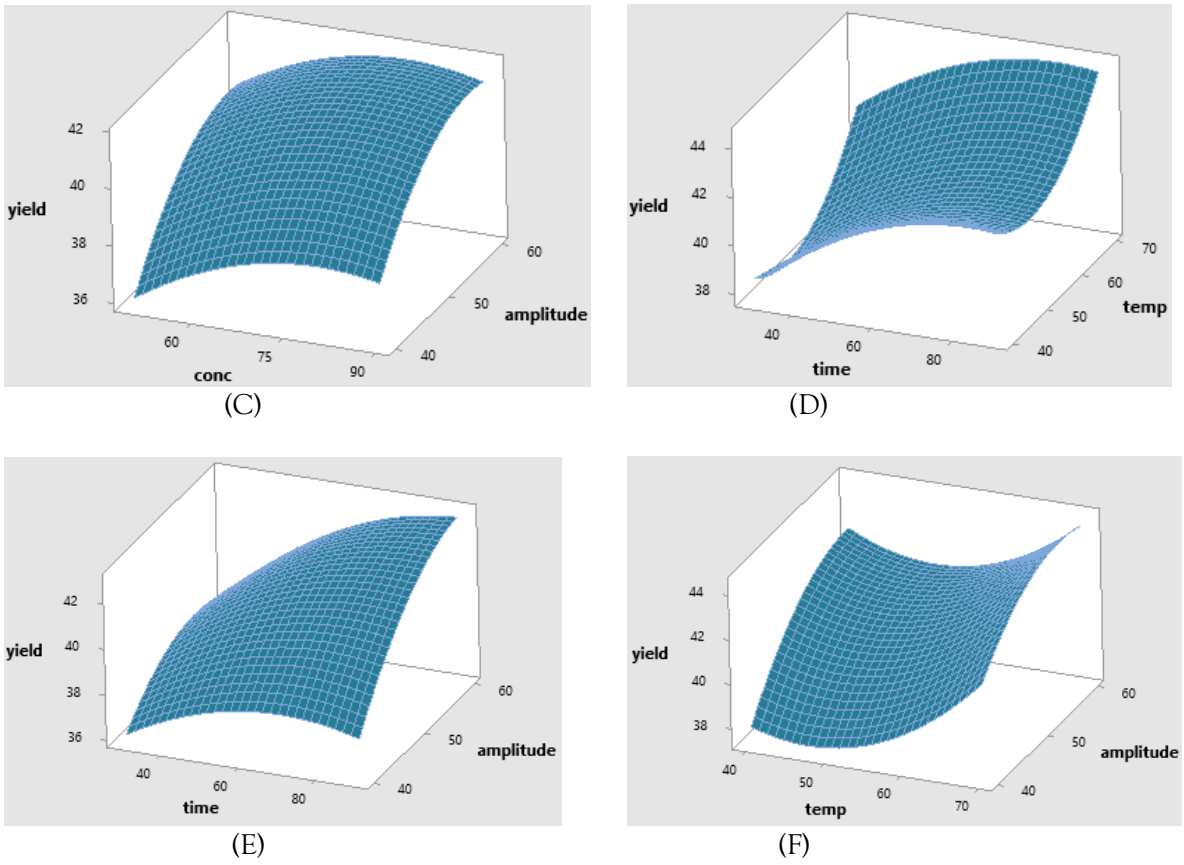
To analyse the relationship among process variables and response variable, three-dimensional response surface plots were generated by varying two independent variables at a time while holding the remaining two variables constant. The surface plots were generated between two independent variables while holding the other two variables constant (Figure 1). Fig. 1A-C demonstrate the effect of interaction between solvent concentration (50-90% ethanol) and three other process variables (time, temperature and amplitude) on the extraction yield of jamun seed extract. The graphs suggested that ethanol concentration has a linear and quadratic effect ($p<0.05$) on the yield of jamun seed extract (Table 3). Having a good solubilizing capacity, ethanol could promote the extraction of polyphenols as well as water causes swelling of cell materials. Consequently, interface between the solvent and plant material increases which results in increased yield for extraction (Hayat et al., 2009). Fig. 1D depicts the combined effect of extraction time and temperature on the response variable. The process parameters upon standardization increased the solubility of phenolics and assisted the efficient diffusion of the solvent into the matrix, entailing the dissolution of the components and hence, their accelerated retrieval. Though, an elevated temperature can cause degradation of certain components, shorter duration promotes efficient extraction (Dahmoune et al., 2015). The interaction of extraction time and sonication amplitude significantly affected the extraction yield (Table 3, Fig. 1E). Cavitation is considered as the main factor held responsible for improved extraction efficiency achieved by ultrasonication. High amplitude causes the cavitation, which facilitates the diffusion of solute efficaciously in the solvent by rupturing the cell wall thus enhancing the yield (Hayat et al., 2009). In Fig. 1F, combined effect of temperature and amplitude on the extraction yield of jamun seed was depicted and found to be significant ($p<0.05$) in terms of their interaction.



(A)



(B)



(C) (D)

(E) (F)

Figure 1. Response surface Plots for extraction output (% yield) from jamun seeds from ultrasound-assisted extraction in terms of (A) Solvent concentration and time; (B) Solvent concentration and temperature; (C) Solvent concentration and amplitude; (D) time and temperature; (E) time and amplitude; (F) temperature and amplitude.

Validation of predicted model

The essential part of the experimental study was to estimate the optimum extraction process conditions to maximize the extraction yield of jamun seed extract. The optimum processing conditions obtained for extraction of jamun seed were 85% ethanol concentration, extraction time 90 minutes, temperature 53℃ and sonication amplitude 50 Hz. The predicted and actual values for the extraction yield were acquired as 43.21% and 43.08% at optimal conditions, correspondingly. It was observed that there existed no significant difference ($p>0.05$) among the predicted as well as experimental values. The findings confirmed that the developed model could provide a precise prediction of the extraction yield.

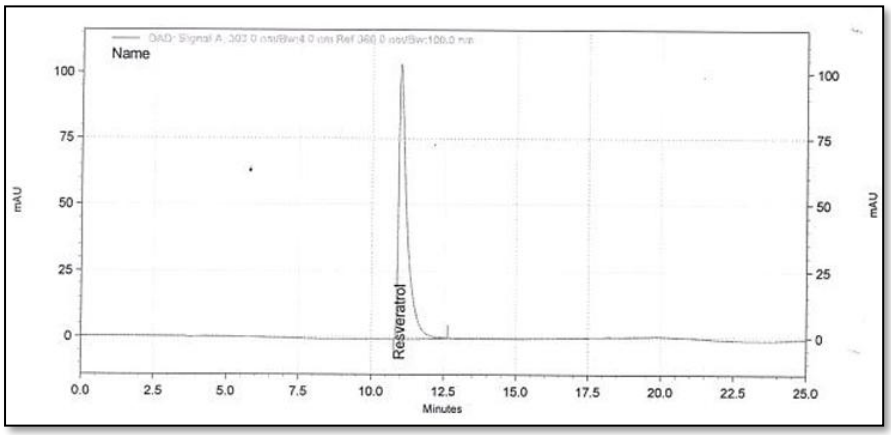
TPC and antioxidant capacity of jamun seed extract

The results obtained for TPC and total antioxidant capacity of jamun seed extract are presented in the Table 4. The antioxidant capacity is defined as the number of moles of free radicals scavenged by a test antioxidant solution; however, this value can differ according to the radical species used in the assay. Because of disparities in reactivity of different kind of reactive oxygen species (ROS) or reactive nitrogen species (RNS), there is no single protocol available to provide an accurate measure of antioxidant potential. Therefore, assessment of the antioxidant ability of the extract was carried out employing three different assays: DPPH, ABTS and FRAP. These methods are generally applied to measure the antioxidant capability of a sample, regardless the mechanism of action is diverse. DPPH and ABTS assays measure sample´s antiradical activity, and FRAP measures its reducing potential. The total phenolic content in jamun seed extract was 315.92 mg GAE/g seed extract, signifying high levels of phenolic compounds. The high TPC may be ascribed to the occurrence of other similar compounds like stilbenes, flavan-3-ols, and proanthocyanidins naturally copious in seed extracts. Accordingly, the seed extract demonstrated remarkable antioxidant activity as determined by different assays with the DPPH, ABTS and FRAP values were 1326.68 mmol TE/g, 1803.27 mmol TE/g and 1174.16 mmol TE/g respectively. A positive relationship was studied for TPC and antioxidant capacity, indicating that phenolics are responsible for antioxidant behaviour. Similar trends have been observed in previous studies on different seed extracts by Jayaprakasha et al [17] and Di Stefano et al. [18], who linked high phenolic content with greater radical

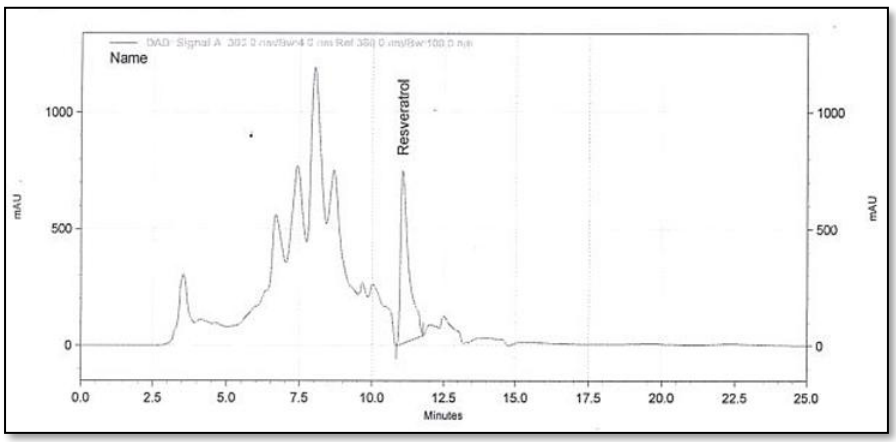
scavenging ability. Differences in the studies may be attributed to variations in extraction approach, fruit variety, environmental conditions, and fruit maturity.

Determination of resveratrol by HPLC analysis

HPLC analysis was performed for detection and quantification of resveratrol content in jamun seed extract. Figure 2a shows the chromatogram of the resveratrol standard and it gave the retention time of the compound which was used to identify the resveratrol in the sample chromatogram. The UV detection at 303 nm aided in good sensitivity and selectivity. Among the phenolic compounds of jamun seed extract, resveratrol was eluted at 11.047 minutes as verified by comparison with the reference standard using HPLC-DAD signal. The resveratrol content in the jamun seed extract in the optimized RSM based ultrasound extraction conditions was found to be 36.27 mg/g dry weight. The UV detection at 303 nm aided in good sensitivity and selectivity. The amount was observed to be higher than that reported by Shrikanta et al. [6] and this could be due to different extraction procedure including time, temperature, solvent concentration as well as variety of fruit used for extraction. Similar results have been reported in previous studies by Guo et al. [19] and Jin et al. [20], who also demonstrated better extraction yields of resveratrol using ultrasound from grape skin and peanut skin, respectively. Moreover, the resolution and sharpness of the peak in the chromatogram indicated accurate and efficient separation. These results establish the efficacy of HPLC as a robust technique for the precise estimation of resveratrol in plant matrices.



(a)



(b)

Figure 2. HPLC Chromatograms of standard resveratrol (a); jamun seed extract (b).

CONCLUSION

The current study developed an improved and optimized procedure for extracting polyphenols from jamun seed using ultrasound assisted extraction (USAE) method. A full factorial Central Composite Design was employed to evaluate the extraction of phenolic compounds from jamun seed. It was observed that the optimum point for best combination of process variables for increasing the extraction yield (response variable) was at 85%, 90 minutes, 53℃ and 50 Hz. It was observed that predicted and experimental data were similar and exhibited no significant difference ($p>0.05$). The obtained extract was

analysed for its TPC and antioxidant capacity. The identification and quantification of resveratrol in jamun seed extract was performed using HPLC analysis. Thus, the results of current study would be useful in extracting resveratrol from an industrial waste resource having environmental, ecological, and potential medicinal benefits. Overall, in terms of extraction from natural plants, subcritical water extraction had the advantages of efficiency, low cost, and being environmentally friendly. However, the seed extract contains more bioactive components. Therefore, a study on the purification of resveratrol as extracted by ultrasonication necessitates further exploration.

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Conflict of Interest

The author(s) declares no conflict of interest.

Authors' Contribution

Preeti Kundu: data curation; formal analysis; investigation; methodology validation; writing-original draft; writing-review & editing. Dr. Jyotika Dhankhar: Conceptualization; project administration; supervision; writing-original draft; writing-review & editing.

Data Availability Statement

The manuscript incorporates all datasets produced or examined throughout this research study.

Ethics Statement

The document accurately and thoroughly presents the authors' original research and analysis.

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