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Original article

Extraction, development and validation of HPLC-UV method for rapid and sensitive determination of colchicine from *Colchicum autumnale L. Bulbs*

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ABSTRACT

Colchicum autumnale L. also known as the autumn crocus, contains colchicine alkaloid having antifungal properties. The tuber of this plant is rich in terms of colchicine. In this research an ultrasound-assisted extraction (UAE) method was optimized for the extraction of colchicine from *Colchicum autumnale L.* bulbs before high-performance liquid chromatography with UV detection (HPLC-UV). Optimization of various extraction parameters was performed using response surface methodology (RSM) to evaluate the maximum colchicine yield from *Colchicum autumnale L.* bulbs. The Box-Behnken design (BBD) and RSM were used to investigate the effect of three key parameters (extraction time (20–60 min), extraction temperature (40–80 °C) and ultrasound power (500–700 W) on extraction efficiency. The variance analysis suggested that the dependent response variable of yield of colchicine may be expressed by a quadratic polynomial model. The optimal theoretical extraction conditions were found to be an ultrasonication power of 602.4 W, an extraction time of 42 min and a temperature of 64 °C. Under these conditions, the optimum foreseen yield was 0.237%. The experimental colchicine yield obtained by following the optimized conditions was found to be 0.238%. These values are very well compatible with each other.

1. Introduction

Colchicum autumnale L., also conversant as the autumn crocus, wild saffron and naked lady; contains colchicine alkaloid that has antimitotic properties which can be used for immobilization the mitosis by preventing DNA synthesis and tubulin polymerization (Folpini and Furfori, 1995). Colchicine, the main alkaloid of *Gloriosa superba*, is used in acute gout attacks, familial Mediterranean fever and liver cirrhosis. Colchicine and the like are used clinically in the treatment of certain leukemia and solid tumors (Kershenobich et al., 1988).

Extraction is the first step to recover and purify the active components of the plant material. There are many techniques for extracting colchicine from members of the colchicaceae family. Among these, the most commonly used methods are soxhlet (Pandey and Banik, 2012) and solid-liquid absorption (Husek et al., 1989). Members of the colchicaceae family, such as *Sandersonia aurantiaca, Colchicum autumnale, Androcymbium melanthioides, colchicine*, are reported to be extracted with methanol (Finnie and Van Staden, 1991), *G. superba* with aqueous methanol (Kannan et al., 2007) and ethanol (Ellington et al., 2003). The effect of extraction is influenced by many parameters, such as solvent type, number of steps, pH, temperature, liquid/solid ratio and particle size of the plant material (Shi et al., 2005).

The Response Surface Method (RSM) is a mathematical and statistical method that can be used to obtain optimal parameters with a small number of experiments. It evaluates the individual and interactive effects of different factors, the interaction of many experimental parameters simultaneously, and predicts the outcome of the variables in the pre-defined condition. The greatest advantage of the RSM is the reduction of the number of

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experiments required to evaluate multiple parameters and their interactions, which lets less time needed to optimize a transaction than other approaches. The RS methodology originally defined by Box and Wilson (1951), is currently successfully used to improve, develop and optimize processes (Xu et al., 2016; Zhou et al., 2017; Bulduk and Sağlam, 2015). Various techniques such as voltametric (Bersier and Bersier, 1994), radio and enzyme immunoassays (Poulev et al., 1994), spectrophotometry (Singh et al., 2000), and various chromatographic (Watterson et al., 2014), methods has been developed to identify and determine colchicine at trace levels. Active ingredients in plants are purified by extraction. Although Soxhlet and solid–liquid extraction are the most commonly used techniques, there are disadvantages such as high cost, excessive time consumption, sample waste, need for toxic organic solvents, and need of large quantities of samples (Talebianpoor et al., 2014).

Ultrasonic assisted extraction has attracted considerable attention recently in the extraction process of the various analytics in different matrices (Fan et al., 2012). Ultrasonic assisted extraction is a cost effective, simple and effective alternative to conventional extraction techniques. It has been proposed as an eco-friendly method as an alternative procedure for sample pretreatment in a shorter time, at lower temperatures. It uses less organic solvent in significant quantities. It has easier manipulation and shorter reproducibility properties (Ma et al., 2011). The usual method to determine optimal conditions in extraction process is keeping all parameters constant, while only variable is the time. This method neglects the interaction between the variables. In recent years RSM has been widely used in statistical experimental design optimization experiments (Xu et al., 2016; Zhou et al., 2017; Bulduk and Sağlam, 2015). The BBD and RSM method can optimize all parameters collectively to remove the limitations of a single-factor optimization process altogether. The objective of this study was to develop a simple, sensitive, precise and reproducible UAE–HPLC–UV method, capable of responding to the demand for cost effective analysis of colchicine from tuber of *Colchicum autumnale L.* with high accuracy and precision.

2. Material and methods

2.1. Preparation of plant material

Colchicum autumnale L. was collected from the Surmene, Trabzon, Turkey in May 2017. Its bulbs were rendered and dried at the temperature of 80 °C in an oven. Dried bulbs were ground to the size of 80–100 mesh before extraction. Colchicine is United States Pharmacopeia (USP) Reference Standard by Sigma-Aldrich. All chemicals used in all experiments were in analytical quality and in all solvent HPLC grades used for chromatographic purposes. 0.45 μm membranes (Millipore, Bedford) were used for the filtration of all solutions (Bulduk and Sağlam, 2015).

2.2. Analytical procedures

2.2.1. HPLC analysis

Identification and quantitative determination of the colchicine in the extracts were carried out by an Agilent brand 1260 model HPLC apparatus. The chromatographic system was equipped with an autosampler, a quaternary pump, a column compartment and a UV–VIS detector. Chromatographic analysis was carried out using a single-column isocratic reverse phase method. Separation was performed by ACE 5 C-18 column (250 mm × 4.6 mm id, 5 μm particle size).

During the mobile phase 450 mL of 6.8 g/L solution of potassium dihydrogen phosphate and 530 mL of methanol were mixed. After the temperature of the mix had cooled down to room temperature, the volume of the mix was completed to 1000 mL with methanol. The pH of the final solution was adjusted to 5.5 with dilute phosphoric acid and filtered through 0.45 μm Millipore filters.

The flow rate the mobile phase was 1.0 mL/min and the injection volume was 20 μL. The column temperature was kept at 30 °C and detection was carried out at 254 nm.

2.2.2. Analytical method validation

The method has been validated according to ICH guidelines, taking the recommendations of other appropriate guidelines into account in terms of precision, linearity, accuracy and stability. The results obtained by testing various parameters during the validation of the analytical method are given in Table 1.

2.2.3. Standard solutions and calibration curve

A standard stock solution of colchicines and an aqueous solution of colchicine at 1000 μg/ml of final concentration were prepared. Standard solutions at concentrations of 100, 200, 300, 400 and 500 μg/ml were prepared by diluting the colchicine stock solution with water. The calibration curve was prepared over a concentration range of 100–500 μg/ml for colchicine with five concentration levels. Linearity for colchicine was plotted using linear regression of the peak area versus concentration. The coefficient of correlation (R2) was used to judge the linearity. The detection limits (LOD) and quantification limits (LOQ) for tested compound were determined by the signal to noise (S/N) ratio (Table 1).

2.3. UAE extraction procedure

Precisely 1.0 g of dried and ground sample was placed into a round bottom flask. 30 mL of 0.1 M hydrochloric acid was added. Ultrasound-assisted extraction (UAE) was performed using a Bandelin Sonorex ultrasonic bath with a frequency of 50 kHz. Erlenmeyer bottles were placed in an ultrasonic bath for standard ultrasonic conditions. The solvent levels in the Erlenmeyer flask and the water level in the ultrasonic bath were kept the same. The temperature and time value of the ultrasonic bath was set and extraction was carried out. The leave extracts were filtered through Whatman filter paper and then filtered with 0.45 μm membrane filter following the extraction procedure (Millipore, Bedford).

2.4. Computation and data analysis for RSM

Response surface methodology (RSM) was used to optimize extraction parameters of the extraction process (Bulduk and Sağlam, 2015; Fan et al., 2012; Ma et al., 2011). The optimization was carried out according to the Box-Behnken design (BBD) with 3 variables at 3 levels on the yields of colchicine by the Design-Expert software (Trial Version 8.0.6). Based on our previous

Table 1
Validation parameters.

Parameters		Colchicine
Specify	Peak Purity Ratio	0.0010
Linearity	Concentration Range of Colchicine Standards ppm	100–500
	Correlation Coefficient of Linearity Equation	0.9996
	Intercept of Linearity Equation	57.68
	Slope of Linearity Equation	21.53
Limit of Detection (ppm)		3.3731
Limit of Quantification (ppm)		7.7390
Retention Time min.		9.480

single-factor experiments, extraction time, ultrasound power and extraction temperature were chosen as independent variables with ranges of 20–60 min, 500–700 W, and 30–70 °C respectively. Data from the BBD were analyzed by multiple regressions to fit the quadratic model. The second-order model equation for each response was as follows:

$$Y = \beta_0 \pm \sum_{i=1}^{3} \beta_i X_i \pm \sum_{i^{\circ}=1}^{3} \beta_{ii} X_i^{\,2} \pm \sum_{i=1}^{2} \sum_{i=2}^{3} \beta_{ij} X_i X_J \quad (1)$$

where Y: predicted response, β_0: intercept, respectively; β_i, β_{ii}, β_{ij}: regression coefficients of linear, quadratic, and interactive effects, X_i, X_j: independent coded variables that affect the responses.

The encoded levels of the independent variables and the parallel parameter values are given in Table 2. The statistical significance of the model was determined using variance analysis (ANOVA) ($p < 0.05$). The model's competence was determined by evaluating the lack of fit and the coefficient of determination (R^2). Three dimensional (3D) response surface graphs were generated by keeping a response variable constant and changing other variables.

3. Results and discussion

The validation parameters that required analytical method validation are summarized in Table 1.

3.1. Single factor experiment

The influence of some factors such as pH, solvent/sample ratio, ultrasound time and temperature on the extraction efficiency was detected and analyzed.

3.1.1. Effect of pH

The pH of the extraction medium has a large effect on the efficiency of the extraction. Extraction solvents having the pH (1, 4, 7, 10, 13) with hydrochloric acid (HCl) and sodium hydroxide were prepared to investigate the effect of pH on the extraction efficiency. Solvent/material ratio was 30:1 mL/g, ultrasonication time was 40 min, ultrasonication temperature was 60 °C, and ultrasonication power was 600 W. The results are shown in Fig. 1. The highest colchicine yield (0.232%) was obtained with the solvent having the pH of 1. As the pH value increases, the yield decreased continuously. So the pH value chosen for the next step is 1. Colchicine is

Table 2
Response surface analysis experimental data and experimental values.

Run	Ext. Time min X_1	Ext. Temp. °C X_2	Ultr. Power W X_3	Colchicine % Actual	Colchicine % Predicted
1	20	60	500	0.178	0.177
2	20	60	700	0.186	0.181
3	60	60	500	0.196	0.184
4	60	60	700	0.189	0.171
5	40	40	500	0.182	0.155
6	40	40	700	0.174	0.139
7	40	80	500	0.184	0.201
8	40	80	700	0.199	0.208
9	20	40	600	0.196	0.168
10	60	40	600	0.184	0.153
11	20	80	600	0.202	0.212
12	60	80	600	0.214	0.224
13	40	60	600	0.235	0.227
14	40	60	600	0.238	0.227
15	40	60	600	0.236	0.227
16	60	40	600	0.184	0.177
17	60	60	500	0.196	0.181

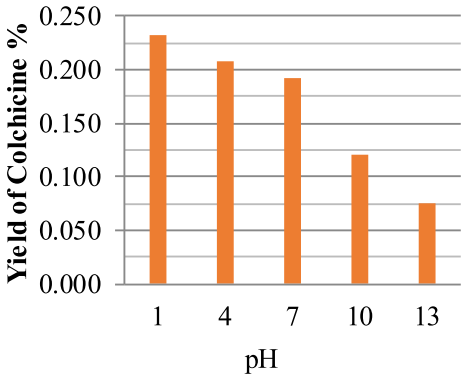

Fig. 1. Effect of the pH on the extraction yield.

an alkaloid and alkaloids generally form alkaloid chloride salts with HCl. Alkaloid chlorides are better soluble in water then base form of alkaloids, and ultimately alkaloids solubility in water is generally low. This is an expected situation. Similarly, in this study, colchicine formed colchicine chloride salt with HCl.

3.1.2. Effect of solvent/material ratio

Different solvent/material ratios were used to study the influence of different liquid-solid ratios on extraction efficiency (15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1 mL/g). pH: 1; The ultrasonication time was 40 min; The ultrasonication temperature was 60 °C, and ultrasonication power was 600 W. The results were shown in Fig. 2. When the solvent/material ratio had increased from 15:1 to 30:1, the extraction efficiency increased by 21% (from 0.182 to 0.231 ± 21.21%). When the ratio of solvent to material was above 30:1, the extraction efficiency had hardly changed at all. The reason is that; when the ratio of solvent to material increased, the mass transfer process can be accelerated and diffusion of the antioxidants into the medium can be facilitated until the mass transfer process reaches the maximum (Xu et al., 2016; Zhou et al., 2017). As a result, the optimum solvent/material ratio was found to be 30: 1.

3.1.3. Effect of ultrasonication time

The effect of different durations of ultrasonication on extraction efficiency was compared, and the results are given in Fig. 3. Extraction conditions were as follows: pH: 1; the ratio of the solvent to the material was 30:1 mL/g; the ultrasound irradiation temperature was 60 °C. Extraction efficiency increased 10 and 40 min and then decreased when the ultrasound irradiation time was longer than 40 min. It was observed that the maximum extraction yield occurred after 40 min. The results demonstrate that solvent-based diffusion of bioactive compounds under ultrasound

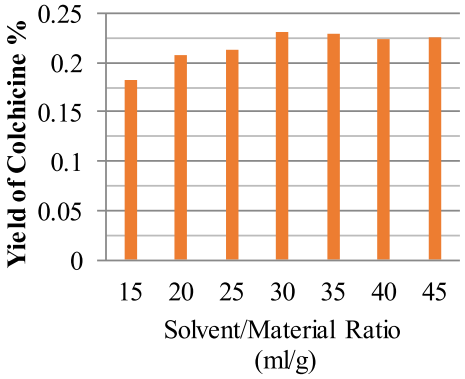

Fig. 2. Effect of the solvent/material ratio on the extraction yield.

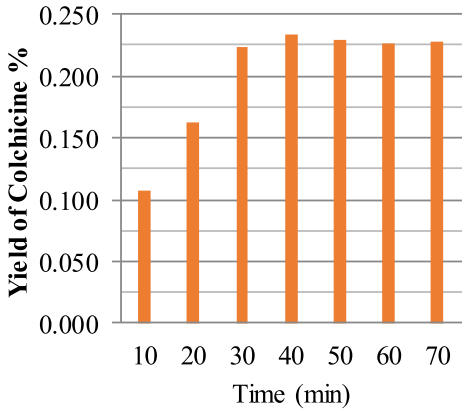

Fig. 3. Effect of the ultrasonication time on the extraction yield.

irradiation treatment can be developed and dissolution balance can occur in a short time. But colchicine may be degraded after prolonged exposure to ultrasonic radiation (Pandey and Banik, 2012; Bulduk and Sağlam, 2015). Therefore we decided to use 40 min in other experiments to be done.

3.1.4. Effect of ultrasonication temperature

The effect of temperature change on extraction yield was investigated and the results were given in Fig. 4. Other extraction conditions were set as; pH: 1; solvent/material ratio is 30:1 mL/g; ultrasound irradiation time is 40 min. Extraction efficiency increased when the temperature was increased from 30 °C to 60 °C, but then decreased when the temperature was raised from 60 °C to 80 °C. It can be said that maximum extraction yield can be obtained at 60 °C with 0.236%. In addition, the results showed that natural colchicine reached desorption and solubility equilibrium at 60 °C, and that colchicine could be decomposed at higher temperatures (Zhou et al., 2017; Bulduk and Sağlam, 2015). Therefore we decided to use 60 °C in other experiments to be done.

3.2. Response surface methodology

Response surface methodology was used to evaluate the interaction of various experimental parameters, as was done in the literature (Khodadoust et al., 2017).

3.2.1. Experimental design and results of BBD

Following the single-factor experiment results, an ultrasound time of 42 min, ultrasound irradiation temperature of 64 °C and ultrasound power of 602.4 W were chosen as the central condition for the Box-Behnken design (BBD) experiment besides, the effects of three independent variables on the value of colchicine as a dependent variable were investigated. 17 different experimental conditions and the results of these experiments are summarized in Table 2. The results showed us the colchicine content of the tuber ranged from 0.174% to 0.238%. The maximum colchicine content was recorded under experimental conditions of 40 min of ultrasound time, 60 °C of ultrasound temperature and 600 W of ultrasonic power.

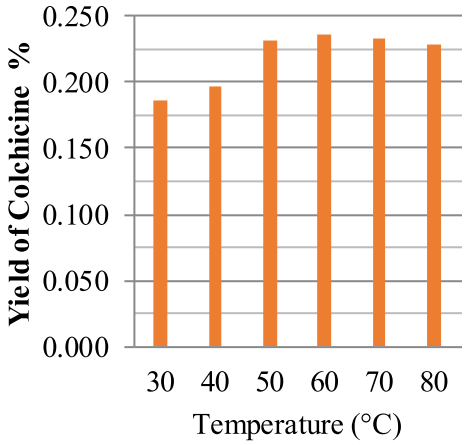

Fig. 4. Effect of the ultrasonication temperature on the extraction yield.

3.2.2. Fitting the model

Variance analysis (ANOVA) for the response quadratic model was performed and results are shown in Table 3. A second-order polynomial model was obtained for the extraction of the colchicine. A statistically significant model with $p = 0.0023$ and satisfactory coefficient of determination is $R^2 = 0.972$. The linear parameters of X_1, X_2, X_3 and quadratic parameters of X_1^2, X_2^2, X_3^2 were significant at the level of $p < 0.01$, while interaction parameters of X_1X_2, X_1X_3 were significant at the level of $p < 0.05$. Lack of Fit-Value when p value = 0.508 was not significant. Significant regression and non-significant lack of fit showed that the regression equation is sufficient to show the real relationship between the response values (Y) and the three independent variables. The second order regression equation was obtained as follows:

$$\begin{aligned} Y\% = & -1.08034 + 0.00391429 \times X_1 + 0.00416519 \times X_2 \\ & + 0.00365458 \times X_3 + 0.0000164327 \times X_1 \times X_2 \\ & - 0.00000213654 \times X_1 \times X_3 + 0.00000287500 \times X_2 \\ & \times X_3 - 0.000043604 \times X_1^2 - 0.0000511619 \times X_2^2 \\ & - 0.00000311186 \times X_3^2 \end{aligned} \quad (2)$$

3.2.3. Response surface analysis

All of the response surface plots were drawn and given in Figs. 5–7. The interactions between various factors may be seen easily from the surface response graph. The effect of ultrasound temperature and ultrasound time on the extraction yield is shown in Fig. 5 (at a constant ultrasound power of 602.4 W). An increase of ultrasound temperature (X_2) resulted in an increase of extraction yield to a maximum at a certain level, while an increase of ultrasound time (X_1) resulted in an initial increase of extraction yield and then decreased as the ultrasound time continued to increase.

The effect of the ultrasound power and ultrasound temperature on the extraction efficiency at a fixed solvent/material ratio of 30:1 mL/g is shown in Fig. 6. In Fig. 5, it can be observed that the ultrasonic power produces similar effects on the extraction efficiency; while, there is a limited effect of the ultrasound time on the extraction efficiency.

The effect of the interaction of ultrasound power and ultrasound time on the extraction efficiency at a fixed ultrasound temperature of 602.4 W is shown in Fig. 7. Looking at the figure we can say that the extraction efficiency has a strong positive effect on the solvent/material ratio, while ultrasound time has a lesser effect. The combination of the ANOVA in Table 3 and response surfaces in Figs. 5–7 suggest that there is a statistically significant relationship between ultrasound time and ultrasound temperature (X_1X_2), and ultrasound time and ultrasound power (X_1X_3), while the relationship between ultrasound temperature and ultrasound power (X_2X_3) is insignificant. This concludes that temperature has a higher effect on ultrasound time. Consecutively, the ultrasound time and temperature have a greater effect on extraction efficiency than ultrasound power.

Table 3
Variance analysis (ANOVA) for the response quadratic model.

Source	Sum of squares	dF	Mean square	*F* Value	*p* value	Significance
Model	0.03300	9	0.00368	19.24	0.0023	Significant
X_1	0.00961	1	0.00961	50.21	0.0009	Significant
X_2	0.00565	1	0.00565	29.54	0.0029	Significant
X_3	0.01000	1	0.01000	53.00	0.0008	Significant
X_1X_2	0.00255	1	0.00255	13.33	0.0147	Significant
X_1X_3	0.00203	1	0.00203	10.58	0.0226	Significant
X_2X_3	0.00009	1	0.00009	0.47	0.5228	Not significant
X_1^2	0.00618	1	0.00618	32.30	0.0023	Significant
X_2^2	0.01100	1	0.01100	56.61	0.0007	Significant
X_3^2	0.00995	1	0.00995	52.00	0.0008	–
Residual	0.00096	5	0.00019	–	–	–
Lack of Fit	0.00092	3	0.00031	18.86	0.0508	Not significant
Pure Error	0.00003	2	0.00002	–	–	–
Cor Total	0.03400	14	–	–	–	–
R-Squared	0.9719	–	–	–	–	–
Adj R-Squared	0.9214	–	–	–	–	–

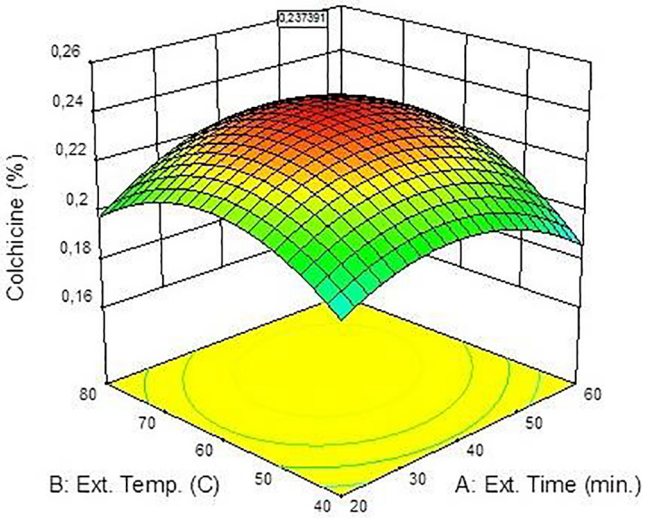

Fig. 5. The effect of ultrasound temperature and ultrasound time.

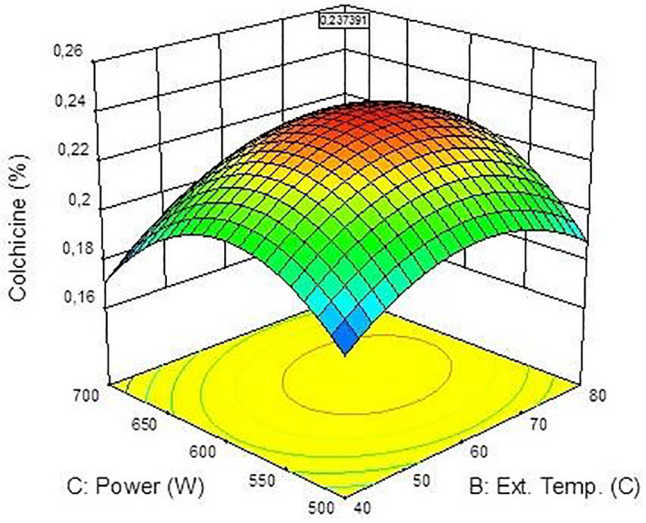

Fig. 7. The effect of ultrasound power and ultrasound time.

Fig. 6. The effect of the ultrasound power and ultrasound temperature.

Also, the chromatogram of a standard solution of colchicine and extract of *Colchicum autumnale L.* are given in Figs. 7 and 8. Chromatogram of colchicine standard solution is 9.48 and the extract of *Colchicum autumnale L.* retention time of colchicine is 9.55 min (see Fig. 9).

3.2.4. Verification of estimated value of the models

Optimum extraction conditions were determined by quadratic polynomial regression model analysis. The most suitable conditions obtained with the model used are as follows: Ultrasonication extraction time: 42.3 min, ultrasonication extraction temperature: 64.4 °C, ultrasonication extraction power: 602.4 W and solvent/-material ratio: 30:1. In optimal conditions a maximum response value of 0.237% was predicted for the model used. Verification experiments were conducted under predicted conditions. So, the adequacy and validity of the obtained regression models were confirmed. In addition, the HPLC method was used to measure the content of colchicine in the extract. As seen from the results given in Table 4, the experimental value is 0.238%, n = 5 and it is consistent with the predicted value. There is a good correlation between

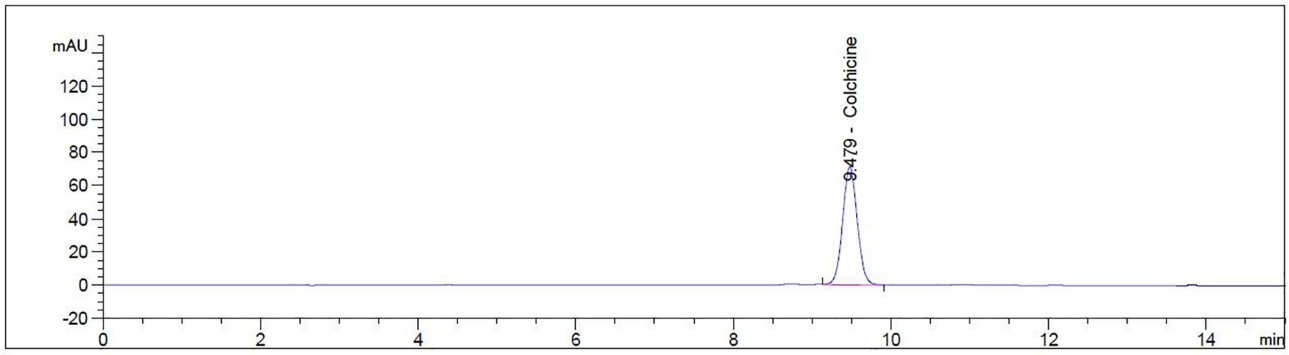

Fig. 8. The HPLC chromatogram of colchicine standard.

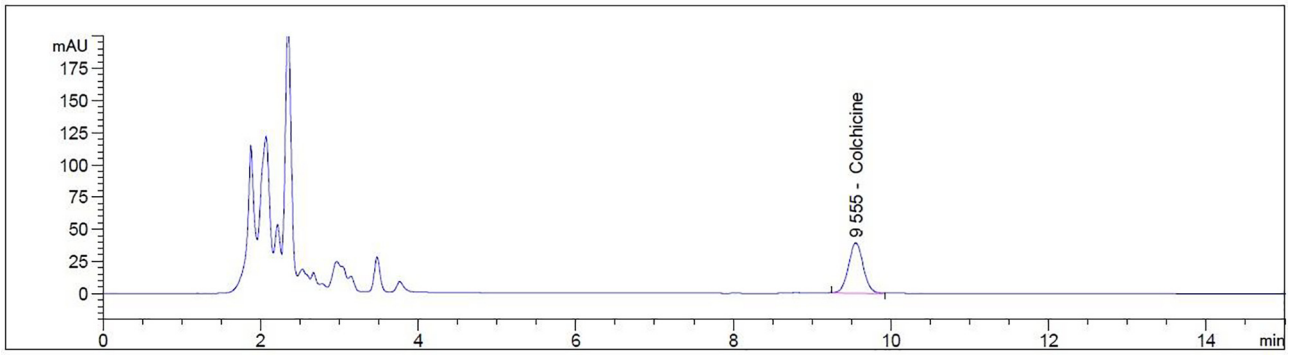

Fig. 9. The HPLC chromatogram of extract of *Colchicum autumnale L.* bulbs.

Table 4
Optimal extraction conditions, predicted and experimental results.

Optimal conditions			Colchicine content	
Extraction Time	Extraction Temperature	Ultr. Power	Experimental Result	Predicted Value
42.3 min	64.4 °C	602.4 W	0.238%	0.237%

the predicted and the experimental results, which indicates that the response surface methodology is an accurate and reliable method of finding optimal conditions of ultrasound extraction.

4. Conclusions

All statistical indications in this study support RSM (Response Surface Methodology) is a successful tool to identify the ultrasonic extraction process of *Colchicum autumnale L.* bulbs for the following tested ultrasonic parameters; power (500–700 W), time (20–60 min) and temperature (40–80 °C) at a frequency of 50 kHz. The dependent response variable represented by the extract colchicine yield can be expressed by a quadratic polynomial model according to the variance analysis and the regression coefficients.

The optimum theoretical extraction conditions were found as follows: ultrasonication power: 602.4 W, extraction time: 42.3 min and extraction temperature: 64.4 °C. The predicted optimal yield of extracted colchicine under the optimal conditions was 0.237%. The actual experimental value of extracted colchicine yield was 0.238% under these conditions. All these results demonstrate the utility of the quadratic polynomial model derived to represent the ultrasonic assisted extraction of colchicine for the variables within the study ranges.

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