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Optimization of Silymarin Extraction Condition from *Silybum marianum* (L.) Gaertn and Development of HPLC Method for Its Quantification

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Abstract
Silybum marianum (milk thistle) and its derivatives are used in the treatment of many diseases, especially liver diseases. In this study, extraction variables required for the industrial process of *Silybum marianum* (L.) Gaertner, and conditions for silymarin extraction were optimized using RSM (Response Surface Methodology). For this purpose, the fixed oil content was determined to be approximately 27% in the extraction using hexane in a Soxhlet apparatus with fruits of certain particle size. An HPLC technique was devised and approved to determine the amount of silymarin in the extract. The HPLC method's linearity was assessed using solutions containing 5 to 30 µg ml^{-1} silymarin. A selective, rapid, accurate, precise chromatographic method was devised and approved to determine the amount of silymarin in plants. Using an Agilent Extend C18 column (250 x 4.6 mm, 5 µm) column, chromatographic separation was carried out. Ultrapure water containing 0.1% formic acid and acetonitrile (67/33, v/v) were used as mobile phase at a flow rate of 1.0 ml min^{-1}. Eluents were detected at 288 nm using a UV detector. The results show that Fed the liquid chromatographic method was linear, precise, accurate, robust, with RSD values below 1.00% and recovery percentage within the standard limits (99-101%). The extracted silymarin yield was calculated to be 0.237% under finest settings.

Research Article

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1. Introduction

The use of medicinal plants as drugs in folk and traditional medicine practices dates back to ancient times. Since prehistoric times, thousands of plants have been utilized to heal a variety of diseases (Kaur et al., 2011; Karkanis et al., 2011). The earliest written evidence of the use of medicinal plants in medicine making was found in a Sumerian clay slab, about 5000 years old, found in Nagpur. It contained 12 recipes for preparing medicines, referring to more than 250 different plants, some of which were alkaloids such as poppy, henbane and mandrake (Petrovska, 2012). It is estimated that 75% of the population in developing countries use natural products, compared to around 50% in developed countries, often linked to lifestyle-related diseases (Lopes et al., 2018). *Silybum marianum* is a medicinal plant whose seeds and fruits have long been used as an alternative medicine due to its hepatoprotective effects. Silybum marianum, a member of the Asteraceae (Compositae) family, gets its name from the white veins that adorn its leaves (Wang et al., 2000). Although it originated in the Mediterranean area, it is also grown as a vegetable in Southern Europe (Ball and Kowdley, 2005). Common names are Milk thistle, Mary thistle, Holy thistle, Silymarin (Kaur et al., 2011). Milk thistle (*Silybum marianum* L.) is a yearly or biennial herb with reddish-purple flowers in July and August (Bijak, 2017). Milk thistle (*Silybum marianum*) is widespread in the Mediterranean area but now common worldwide. Its main stem is robust, protruding with branching. The seeds of *Silybum marianum* are ¼ inch lengthy, flat, smooth, and glossy with a varied black to brown tint (Kaur et al., 2011) (Figure 1).

Figure 1. Milk thistle (*Silybum marianum* L. Gaernt.) and its fruit (Bijak, 2017)

In the 1970s, the WHO recognized silymarin from the seeds of *Silybum marianum* (L.) as an official drug with hepatoprotective properties (Bijak, 2017). Heparmed tablet, Cardo Mariano capsule, Silymarin capsule, Artichokeplus combined capsule, MDA capsule, Hangover capsule, Hepaminol capsule, Silimar tablet, Milk Thistle-Solgar capsule, Milk Thistle-Arkopharma capsule, MilkThistle-Balen capsule, Milk Thistle-Natures Bounty capsule, Hepa-4 combined tablet are the Turkish preparation of milk thistle (Wallace et al., 2003).

Silymarin contains three flavonoid isomers: silybin, silydianin, and silychristin (Karkanis et al., 2011). Silybin is the most biologically active molecule and includes 50-70% silymarin (Ramasamy and Agarwal, 2008) as well as several flavonolignans, including isosilybin, dehydrosilybin, desoxysilycristin, desoxysilydianin, silyandrin, silybinome, silyhermin, and neosilyhermin (Kvasnička et al., 2003) (Figure 2).

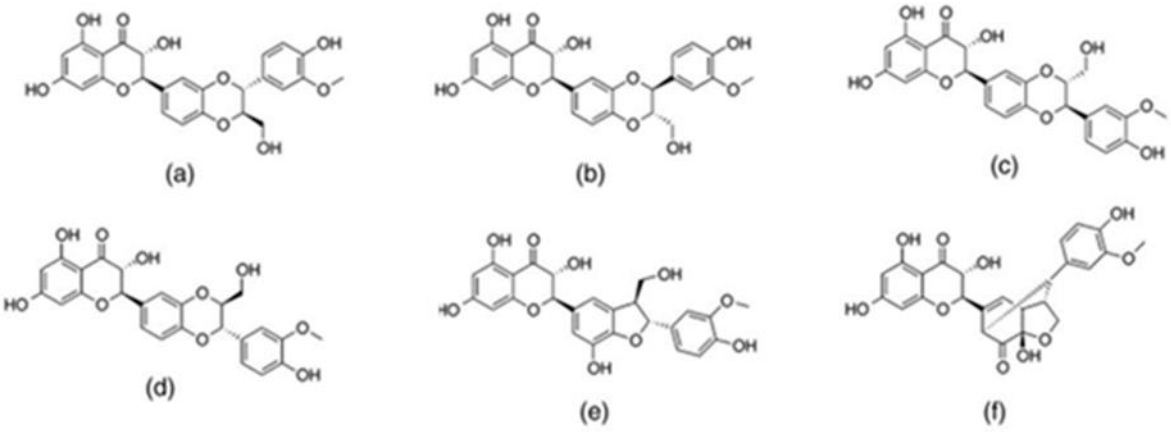

Figure 2. Chemical structures of main flavolignans contained in silymarin, namely, (a) silybin A, (b) silybin B, (c) isosilybin A, (d) isosilybin B, (e) silychristin, and (f) silydianin (Abenavoli et al., 2018)

In this study, *Silybum marianum* seeds, which were ground to a certain size and moisture content was determined, were first defatted in the Soxhlet apparatus using petroleum ether and hexane. After determining the appropriate solvent for the degreasing process, the degreased seeds were extracted using ultrasound-assisted extraction method. HPLC technique was applied using RSM (Response Surface Methodology) to determine the amount of silymarin, the active ingredient of *Silybum marianum* and the optimum conditions for extraction in the extract.

2. Materials and Methods

The dried Silybum marianum fruits used in the investigation were provided from Istanbul University, Faculty of Pharmacy The method of investigation based on study by Dranik et al. (1992). First of all, the fruits were fractionated with ASTM sieve series and ground so that the fraction passing through 0.841 mm and not passing through 0.425 mm could be used in the experimental studies and degreasing process was applied after moisture determination.

2.1. Moisture determination

Since the findings of the investigations should be given on a dry basis, the ground fruits were dried in Shimadzu Libror EB-280 MOC model electronic moisture determination device at a temperature of 105 ^{0}C until they got a consistent weight. and moisture was calculated.

2.2. Extraction of fixed oil

The elimination of fixed oil was measured via a Soxhlet device with 15-20 g samples and the yield was determined depending on time. In this process, petroleum ether (Merck, 40-60 ^{0}C) and hexane (Merck, anhydrous, 95%) were used as solvents and continued for about 14 hours until all fixed oil in the fruits was consumed.

2.3. Sample preparation

1 g of the dried, ground and defatted fruits of the plant were carefully weighed into 100 ml volume flasks and 50 ml of solvent was added. For the duration of the experimental program, the flasks were put in an ultrasonic bath. A 50 kHz Bandelin Sonorex ultrasonic bath was used to carry out ultrasound-assisted extraction (UAE). Both the duration and the temperature of the ultrasonic bath were adjusted to carry out the extraction procedure.

2.4. Analytical instruments and conditions

Agilent HPLC equipment was used for the analyses. 1260 system consisting of a quad gradient pump, auto sampler, UV detector and Chem Station software. An Agilent Extend-C18 column (4.6 mm x 250 mm, 5.0 μm) was utilized and kept at 25 °C. The eluents were chromatographically detected at 288 nm with a UV detector. The mobile phase was ultrapure water and acetonitrile (67/33, v/v) with 0.1% formic acid at a flow rate of 1.0 mL/min. Spectrophotometric analyses have been carried

out on a Shimadzu UV 1800 dual beam path spectrophotometer (Shimadzu, Japan) with UV-Probe software. Standard solutions were scanned in the UV spectrophotometer to determine λ_{max} in the range 200-800 nm and measurements were obtained blindly against ethanol. The silymarin content was determined at a wavelength of 288 nm.

2.5. Stock standard solutions

Silymarin complex stock standard solution: 25 mg of silymarin complex was accurately weighed into a 50 ml beaker and the silymarin complex was dissolved in 20 milliliters of ethanol, bringing the volume to 50 milliliters overall. As a result, 500 µg mL^{-1} of stock standard solution had been produced.

2.6. Slibinin stock standard solution

20 mL ethanol was added to 25 mg slibin in a 50 mL beaker to dissolve the slibinin. Ethanol was added to reach the total volume up to 50 mL. Thus, a stock standard solution had been made with a 500 µg mL^{-1}concentration.

2.7. Standard solutions

Standard solutions at concentrations of 5, 10, 15, 20, 25 and 30 µg mL^{-1} were made by diluting the stock standard solution of silymarin complex and silybin at a concentration of 500 µg $mL^{-1}$with ethanol.

2.8. Validation procedure

The standard solution at 20 µg mL^{-1} concentration was scanned in UV spectrophotometer (Shimadzu UV-1800 spectrophotometer) in the range of 200-1100 nm. The maximum absorbance wavelength λ_{max} of silymarin was determined as 288 nm from the UV spectrum. Validation parameters (specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy and robustness) were investigated (Chlopcíková et al., 2004; Üstünes, 2011). The new HPLC method was designed for quantification of silymarin in plant materials. Based on these considerations, a specific concentration range was selected for the validation procedure. Accordingly, the concentration range of silymarin for method validation was selected as 5-30 µg mL^{-1}.

2.9. Linearity

Stock standard solutions of both silmarin and slibinin were injected into the HPLC device at six different concentrations of 5, 10, 15, 20, 25 and 30 µg mL^{-1} with ethanol and the peak areas in the chromatograms were recorded. Peak regions were plotted against standard concentrations to create calibration curves. The data collected were assessed by utilizing the least squares approach.

2.10. Precision

Intraday precision tests were assessed by injecting the standard solution at a concentration of (20 µg mL^{-1}) into the HPLC device six times on the same day. The six injections' concentration findings were noted, and the mean, standard deviation, and relative standard deviation values were computed. For inter-day precision tests, the standard solution of the same concentration was injected into the HPLC device six times a day on three consecutive days. Following the recording of the concentration results from the six injections, the mean, standard deviation, and relative standard deviation values were computed.

2.11. Accuracy

Recovery studies evaluated three percent of accuracy (80%, 100%, and 120%) for the analytical procedure. This was carried out via analyzing a sample of known concentration in comparison the measured and "true" values. A well-characterized standard solution (20 µg mL^{-1}) was used. To this standard solution was added (80, 100 and 120% of the standard amount) and analyzed with the developed assay method. For every concentration, three samples were prepared, subjected to high-performance liquid chromatography techniques, and recovery percentages were computed.

2.12. Specificity

A prepared solution of the sample (20 µg mL^{-1}) was injected into the chromatographic equipment to test for interfering peaks. After sample analysis, the chromatograms were evaluated for peak area and herbal impurity

interference during silymarin retention periods.

2.13. Limitations of detection and quantification

The chromatographic method's sensitivity was evaluated using the limits of detection (LOD) and limit of quantitation (LOQ). Using equations (1) and (2), they were computed individually based on the standard deviation of the slope and intercept of the calibration curve.

LOD = 3.3xσ/S (1)

LOQ = 10xσ/S (2)

S: slope of the calibration curve and σ: standard deviation of the y-intercept

2.14. Robustness

To assess the robustness of the suggested HPLC method, the results were examined after slightly altering the procedure settings.

The mobile phase's flow rate (±0.1 mL min-1)

Acetonitrile content in mobile phase (±2%)

Column temperature (± 5 °C)

The mobile phase was analyzed under these conditions with changes such as pH value (± 0.10) and the effect on system suitability parameters was observed.

2.15. Surface response methodology data analysis

The extraction process's extraction parameters were optimized by the application of the technique of response surfaces (RSM). Design-Expert software (Trial Version 8.0.6) was used to optimize silymarin yields using three variables at three different levels in accordance with Box-Behnken design (BBD). Extraction time, ultrasound power and extraction temperature were selected as independent variables in the range of 20-60 min, 500-700 W and 30-70 °C, respectively. Multiple regressions were used to examine BBD results in order to fit the quadratic model. The quadratic model equation for each response is as follows:

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

Where;

Y: expected outcome,

β_0: intercept;

β_i, β_{ii}, β_{ij}: regression coefficients for linear, quadratic and interactive effects,

X_i, X_j: Independently coded variables that influence responses.

The adequacy of the model was assessed by evaluating the incompatibility and coefficient of determination (R2). The three-dimensional (3D) response surface plots were set up by keeping one response variable fixed while altering the other variables.

3. Results and Discussion

3.1. Moisture amount

Moisture determination at 105 ^{0}C revealed that milk thistle (Silybum marianum) seeds contain 4.6% moisture.

3.2. Fixed oil amount

Figure 3 shows the yields calculated as a percentage by weight and plotted versus time by weight on dry matter basis using hexane and petroleum ether in the soxhlelet equipment. As seen in the graph, both solvents are suitable for the removal of fixed oil in the fruit, but hexane extraction rate and capacity are slightly superior to petroleum ether. The fixed oil content, which was determined as approximately 27% in the extraction with hexane, was determined as 25% in petroleum ether.

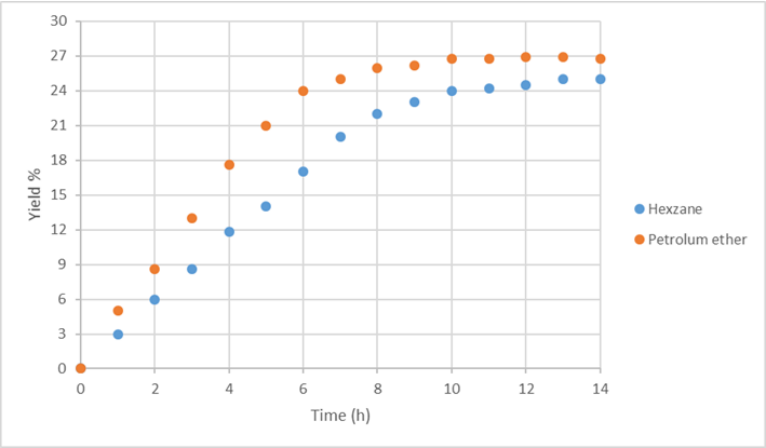

Figure 3. Fixed oil extraction in soxhlelet apparatus

3.3. Method development

Several criteria were used to optimize the chromatographic settings, including the mobile phase's composition, flow rate, pH, and column type. The mobile phase was investigated using various ratios of water:ethanol, water:acetonitrile, and ethanol:acetonitrile. An effective technique for measuring silymarin and silybin in plant materials and pharmaceutical formulations was developed and validated through the optimization of chromatographic settings. In a spectrophotometer, standard solutions of silymarin and silybin at a concentration of 20 µg mL^{-1} in the 200–800 nm range were first scanned against ethanol. A wavelength of 288 nm was chosen for the maximum absorption of silymarin and silybin. The mobile phase was then acidified with formic acid, and flow rates were varied to determine the technique of analysis. A variety of column parameters were examined, and the Agilent Extend C 18 (250 mm × 4.6 mm i.d., 5 µm) column exhibited good peak morphologies (sharp peaks) and good resolution. Due to its many benefits, including its excellent chromatographic peak shape, good column efficiency, and low column pressure, chromatographic analysis was performed for 15 minutes at 30 °C. A mobile phase of water and acetonitrile (67:33, v/v) with 0.1% formic acid, a flow rate of 1.0 mL-min^{-1}, a column temperature of 30 °C, and an injection volume of 20 µL resulted in reasonable retention time and optimum separation. A UV detector with a 288 nm wavelength was used to measure the eluent. Figure 4 shows the chromatogram of the silymarin complex (30 ppm) obtained under these chromatographic conditions. The chromatogram of the silybin standard solution was 7.298 minutes, as shown in Figure 5.

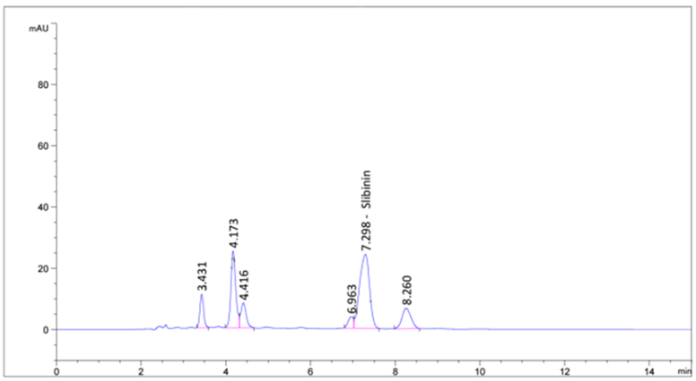

Figure 4. Chromatogram of silymarin complex (30 µg mL^{-1})

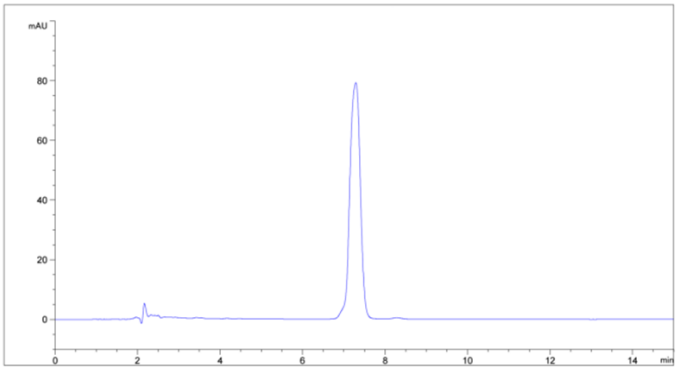

Figure 5. Chromatogram of slibinin standard solution (30 µg mL^{-1})

3.4. Specificity

This method's specificity was assessed by using blank solvents or mediums and then samples that contained the drug slibinin alone. Interference studies were demonstrated by injection of mobile phase, sample and standard solution. There was no interference or peak seen for slibinin with a retention time of 7.298 minutes (Figure 6) (Figure 7).

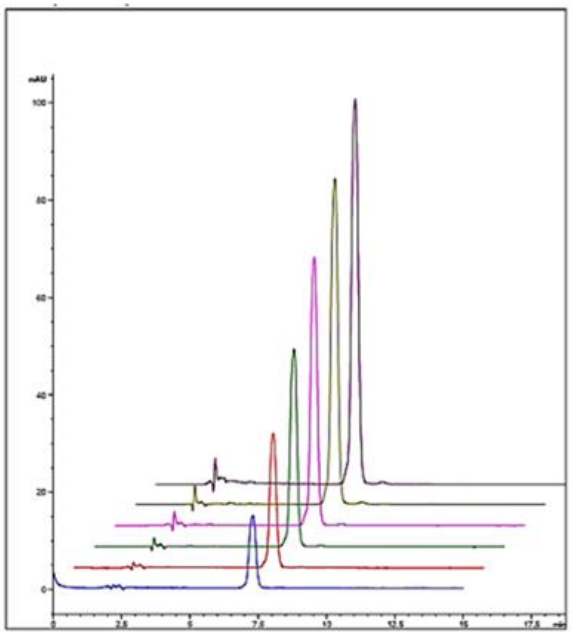

Figure 6. Overlay chromatogram obtained for standard solutions of slibinin (5-30 µg mL^{-1})

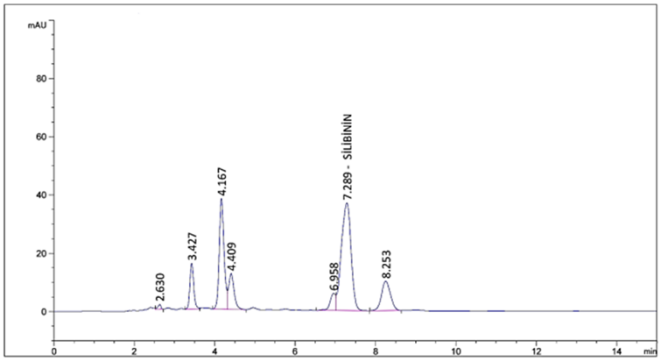

Figure 7. Chromatogram of silymarin complex (20 µg mL^{-1})

3.5. Linearity

To assess linearity, six concentration levels (5-30 µg mL^{-1}) of slibinin solution were prepared. The calibration curves' mean linear regression equation was

$y = 45.229 x - 21.867$ ($r^2 = 0.9999$) (Figure 8)

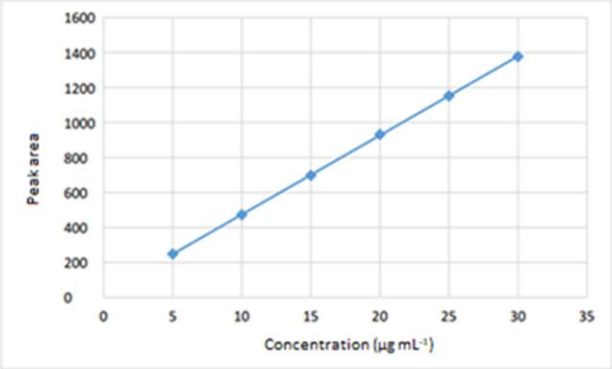

Figure 8. Linearity plot obtained analytically for slibinin (288 nm)

3.6. LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were determined using this equation:

LOD or LOQ = κSDa/b

where 10 for LOQ and κ= 3 for LOD, SDa is the standard deviation of the cut and b is theslope. For slibinin

LOD: 0.90 µg mL-1 and LOQ: 2.70 µg mL^{-1}.

3.7. Accuracy

The proposed method's accuracy was demonstrated using the usual addition technique. Pure sample solution (40 µg ml^{-1}) was added to standard slibinin solutions at concentrations of 5, 15 and 30 µg mL^{-1} and then analyzed. The recovery rates varied from 100.08 to 100.41%. Table 1 shows the data gathered from the recovery study.

Table 1. Accuracy of HPLC method

Nominal Value Slibinin (µg mL^{-1})	Spiked quantity (µg mL^{-1})	Measured quantity (µg mL^{-1})	Recovery (%)	R.S.D.(%)
40	10	50.04	100.08	0.12
	40	80.27	100.34	0.46
	120	160.65	100.41	0.53

3.8. Precision

Precision was reported as relative standard deviation (RSD% = SD/mean×100). The relative standard deviation (RSD) of the calibration standards (n = 9) for intra-day precision (repeatability) (n = 3) and inter-day (intermediate) precision (Table 2) were 0.59% and 1.80%, respectively.

Table 2. Precision of HPLC Method

Compound	Repeatability Intraday (n=3)		Repeatability Inter-Day (n=9)	
	Mean	R.S.D. %	Mean	R.S.D. %
Slibinin	40.07	0.59	40.78	1.80

3.9. System conformity

The system suitability parameters (symmetry factor and retention factor) were calculated for the lowest (5 µg mL^{-1}), middle (15 µg mL^{-1}), and maximum (30 µg mL^{-1}) concentrations for these analytes and are given in Table 3. In this investigation, the calibration curve coefficient exceeds 0.999, showing that the approach is appropriate for samples with simple or very complex matrices. Under optimum conditions, a 0.237% extracted silymarin yield was expected. In these conditions, the extracted silymarin yield's actual experimental value was 0.238%.

Table 3. Results for system suitability

System conformity parameters	Std. Solution Cons. (µg mL^{-1})		
	5	**15**	**30**
Symmetry factor	0.6597	0.6530	0.6576
Peak areas (% RSD)	0.1685	0.1527	0.1432
Detention periods (% RSD)	0.0597	0.0536	0.0521

3.10. Robustness

The results of the robustness analysis demonstrate that minor adjustments to the important method parameters have no effect on the developed method's linearity, absolute mean recovery, or accuracy. The relating results are shown in Table 4. Temperature variations, flow rate, acetonitrile content and pH value did not affect the amount of this analyte recovered. For every compound, the absolute mean recovery ranged from 99% to 101%, with an RSD level of less than 1.00%.

Table 4. Results for robustness

Robustness Parameters	Parameter value	Mean return as a percentage of gain %	R.S.D. %
Mobile phase flow rate	0.90 mL min^{-1}	100.34	0.12
	1.10 mL min^{-1}	99.88	0.05
Column temperature	20 C	99.76	0.07
	30 C	100.21	0.18
Content of acetonitrile in the mobile phase	% 31	100.86	0.93
	% 35	99.91	0.64
pH value	1.90	100.12	0.71
	2.10	99.75	0.63

3.11. Effect of pH on extraction yield

In the extraction process, the pH value has a great influence on the extraction yield. Extraction solvents with hydrochloric acid (HCl) and sodium hydroxide with pH (1, 4, 7, 10, 13) were prepared to investigate the effect on extraction efficiency. The solvent/material ratio was 30:1 mL g^{-1}, ultrasonication time 40 min, ultrasonication temperature 60 °C and ultrasonication power 600 W. Figure 9 shows the results. The highest yield of silymarin (0.232%) was obtained with a solvent with a pH value of 1. The yield decreased continuously as the pH value increased.

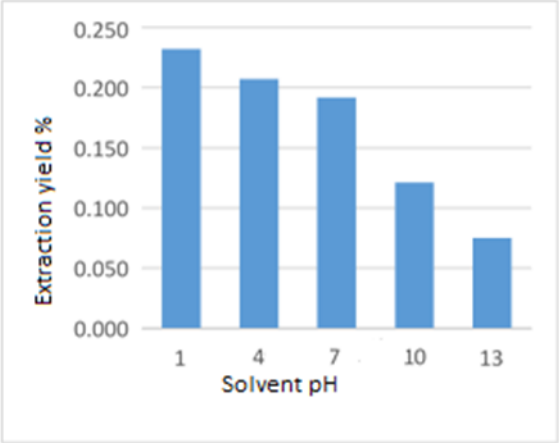

Figure 9. Effect of solvent pH on extraction yield

3.12. Effect of solvent/material ratio on extraction efficiency

To assess the influence of various liquid-to-solid ratios on extraction efficiency, several solvent-to-material ratios were used (15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1 mL g^{-1}), pH: 1; The duration of the ultrasonication was 40 min.; Ultrasonication temperature was kept constant at 60 °C and ultrasonication power at 600 W. The results are given in Figure 10. According to these data, the optimum solvent/material ratio was found to be 30:1.

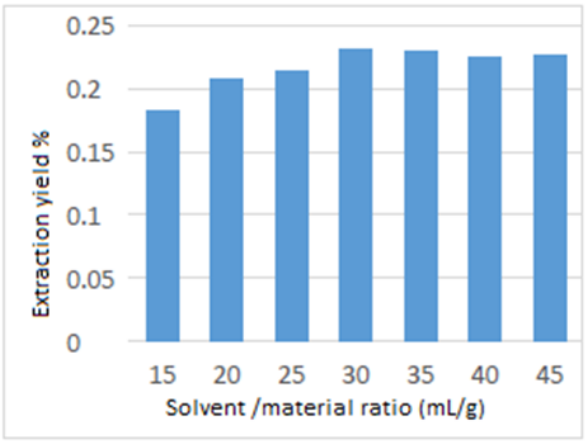

Figure 10. Effect of solvent/material ratio on extraction yield

3.13. Effect of ultrasonication time on extraction yield

Figure 11 shows an evaluation of the effects of various ultrasonication times on extraction efficiency. The extraction effectiveness increased between 10 and 40 minutes, then declined when the ultrasonication time exceeded 40 minutes. The maximum extraction yield was observed to occur after 40 minutes. Conclusions, it shows that solvent-based diffusion of bioactive substances under ultrasonication can be enhanced and dissolution equilibrium can happen rapidly.

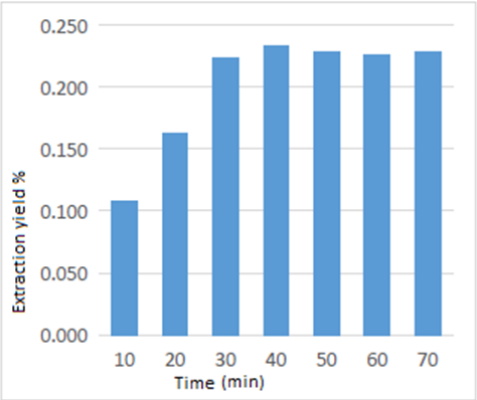

Figure 11. Effect of ultrasonication time on extraction yield

3.14. Effect of ultrasonication temperature on extraction yield

Figure 12 shows the findings of an investigation on the impact of temperature changes on extraction efficiency. Other extraction conditions are; pH: 1; solvent/material ratio 30:1 mL g^{-1}; ultrasound irradiation time 40 min. When the temperature was raised from 30 to 60 °C the extraction efficiency increased; however, when the temperature was raised to 80 °C, the extraction efficiency decreased. It is possible concluded that at 60 °C, a maximum extraction yield of 0.236% is achievable. It was also demonstrated by the data that natural silymarin can degrade at higher temperatures and that it reached desorption and solubility equilibrium at 60 °C.

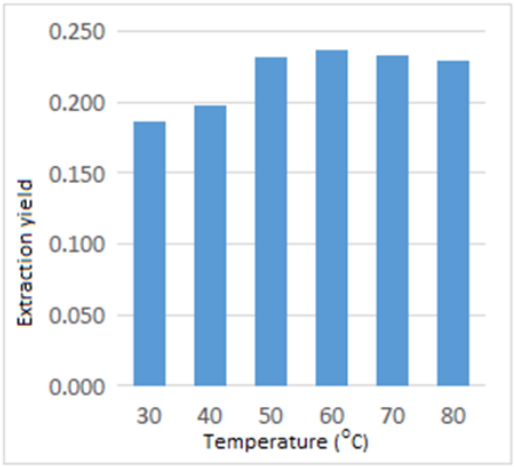

Figure 12. Effect of ultrasound temperature on extraction yield

3.15. The layout of the experiment and the BBD findings (Box-Behnken Design)

Following the results of the single-factor experiment, the center for the Box-Behnken Design (BBD) experiment An ultrasound duration of 42 minutes, an ultrasound radiation temperature of 64 °C and an ultrasound power of 602.4 W were selected as conditions. Independent variables were analyzed on silymarin value as the dependent variable. Table 5 shows the 17 different experimental conditions and their corresponding outcomes. The results showed that the silymarin content ranged from 0.174% to 0.238%. The maximum silymarin content was determined under the conditions of 40 min of ultrasound, 60 0C of ultrasound temperature, and 600 watts of ultrasonic power.

Table 5. Surface response metadology conditions and yield values

Run	Ext. Time (min) X1	Ext. Temperature °C X2	Ultrasound Power W X3	Silymarin % Actual
1	20	60	500	0.178
2	20	60	700	0.186
3	60	60	500	0.196
4	60	60	700	0.189
5	40	40	500	0.182
6	40	40	700	0.174
7	40	80	500	0.184
8	40	80	700	0.199
9	20	40	600	0.196
10	60	40	600	0.184
11	20	80	600	0.202
12	60	80	600	0.214
13	40	60	600	0.235
14	40	60	600	0.238
15	40	60	600	0.236
16	60	40	600	0.184
17	60	60	500	0.196

3.16. Model derivation

Table 6 shows the results of the analysis of variance (ANOVA) carried out for the response quadratic model. A second order polynomial model for silymarin extraction was obtained. p= 0.0023 and a statistically significant model with an acceptable coefficient of determination R2 = 0.972. The linear parameters X1, X2, X3 and quadratic parameters X12, X22, X32 were significant at $p < 0.01$ level, and the interaction parameters X1X2, X1X3 were significant at $p<0.05$ level. The following is the equation for the second order regression that was obtained (Table 6).

Table 6. 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
X1	0.00961	1	0.00961	50.21	0.0009	Significant
X2	0.00565	1	0.00565	29.54	0.0029	Significant
X3	0.01000	1	0.01000	53.00	0.0008	Significant
X1X2	0.00255	1	0.00255	13.33	0.0147	Significant
X1X3	0.00203	1	0.00203	10.58	0.0226	Significant
X2X3	0.00009	1	0.00009	0.47	0.5228	Not significant
X12	0.00618	1	0.00618	32.30	0.0023	Significant
X22	0.01100	1	0.01100	56.61	0.0007	Significant
X32	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	-	-	-	-	-

3.17. Surface response analysis

Each response surface plot has been drawn and the results are shown in Figures 13–15.The impact of ultrasound temperature and duration on extraction yield is shown in Figure 14 (at a constant ultrasound power of 602.4 W). An increase in ultrasonic temperature resulted in a maximum extraction yield at a certain level (X2), whereas Extraction yield initially

increased in outcome of an increase in ultrasound length (X1), but subsequently dropped as the ultrasound duration increased. The ANOVA in Table 6 and Figures 12-14 show that there is a statistically significant relationship between the combination of response surfaces and ultrasound temperature (X1X2), ultrasound duration and ultrasound power (X1X3) while the relationship between ultrasound temperature and ultrasound power (X2X3) is insignificant. This indicates that temperature has a higher impact on the duration of ultrasound. Sequentially, ultrasound duration and temperature have a greater impact on extraction effectiveness compared to ultrasound power.

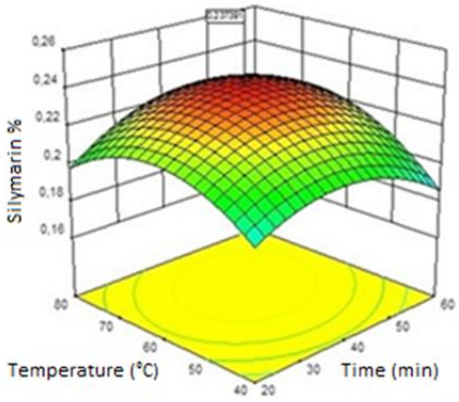

Figure 13. Effect of ultrasound temperature and ultrasound duration on extraction yield

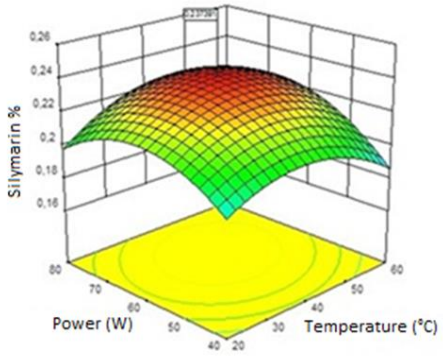

Figure 14. Effect of ultrasound power and ultrasound temperature on extraction yield

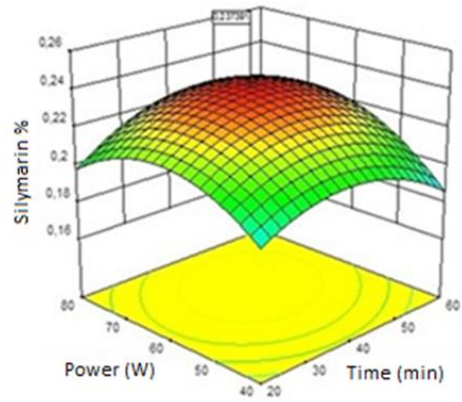

Figure 15. Effect of ultrasound power and ultrasound duration on extraction efficiency

Table 7. Optimum extraction conditions, predicted and experimental values

Optimal Conditions				Silymarin content
Extraction duration (min)	**Extraction temperature (**°C)	**Ultrasound power**	**Experimental value**	Calculated Value
42.3 min.	64.4 °C	602.4 W	0.238	0.237

3.18. Validation of the predictive value of models

A second order polynomial regression model was analyzed in order to identify the ideal extraction conditions with this model. The most appropriate parameters were certained as follows: ultrasonication extraction power of 602.4 W, temperature of 64.4 °C, duration of 42.3 min, and ratio of 30:1 for the solvent to material. Under optimal conditions, a maximum response value of 0.237% was estimated for the model used. Validation experiments were carried out under the prescribed conditions. Thus, the derived regression models' validity and appropriateness were confirmed. The silymarin content of the extract was also determined using the HPLC technique. Table 7 shows that the experimental value was 0.238% (n=5), which is consistent with the estimated to value. The expected and experimental outcomes are significantly correlated. indicating that the response surface approach is a precise and dependable method to find the optimal conditions of ultrasound extraction.

The results of this study indicate that following the extraction process, hexane is used as the solvent for removing oil RSM (Response Surface Methodology) as an effective technique to characterize the ultrasonic extraction process of silymarin from the fruits of the milk thistle plant, for the following examined ultrasonic parameters: power (500-700 W), time (20-60 minutes) and temperature (40-80 °C) at a frequency of 50 kHz. The dependent response variable may be expressed using a quadratic polynomial model based on analysis of variance and regression coefficients, which is represented by extracted silymarin yield. The optimal theoretical extraction conditions were determined as follows: ultrasonication power: 602.4 W, extraction time: 42.3 min and extraction temperature: 64.4 °C. While the expected yield for silymarin extracted under the conditions specified in this study was 0.237%, the actual experimental yield was found to be 0.238%. According to the study's results, a second-order polynomial model can be used to express ultrasonically assisted silymarin extraction for variables in the operating ranges under these experimental conditions.

4. Conclusions

Silymarin, which is one of the components contained in Silybum marianum, has been the subject of many researches in recent years. The major constituent of silymarin, silybin, and its other components are also known to have pharmacological properties. Although silymarin is safe and has several properties that suggest it could be utilized to treat liver illness, such as effects on liver regeneration, lipid peroxidation, inflammation, and hepatic fibrogenesis, data from clinical trial evidence is lacking. Although milk thistle is well adapted to many different habitats and has pharmaceutical and economic value, scientific study is required on the widely used cultivation of this plant as well as extraction and analysis methods for in vitro and in vivo investigations.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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