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Total phenolics and total flavonoids in *Ginkgo biloba* leaves of the plant optimization of the extraction conditions

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Abstract

Due to the negative health effects of artificial antioxidants, consumer interest in natural products has increased in recent years. The importance of natural antioxidants derived from plant sources is gradually increasing in research on the use of antioxidants as preservatives to prevent oxidative deterioration of foods. Free radicals cause degradation reactions in foods. They also cause important problems such as cancer, progeria, and heart disease in living organisms. Eating foods high in antioxidants has an important impact on slowing and stopping health problems. Phenols and flavonoids, known for their antioxidant activity, are found in many medicinal plants and provide various biochemical benefits to living organisms. Many different methods are used to obtain natural antioxidants. Current research is moving in the direction of further developing these methods. In this study, the antioxidant content of *Ginkgo biloba* leaves was investigated. A highly efficient ultrasound-assisted extraction method with short extraction time and minimal solvent consumption was developed for the extraction of *Ginkgo biloba* leaves. Experimental conditions for extraction yield: ethanol concentration 25-100%, solid/solvent ratio 100 mg 30-70 ml-1 sample, extraction time 15-60 minutes, temperature 30-70 ˚C. The result of the experimental study: ethanol concentration: 75%, extraction time: 45 minutes, temperature: 50 ˚C found for the best extraction efficiency. Optimization results for the amount of phenolic substance: extraction time: 31.22 min, extraction temperature: 54.12 °C, ethanol concentration: 57.94%. Optimization results for the amount of flavonoid substance: extraction time: 47.88 min, extraction temperature: 36.34 ˚C, ethanol concentration: 69.51%.

Keywords: Ginkgo Biloba, Phenolic, Flavanoid, Extraction, Optimization

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INTRODUCTION

Traditional medicine has long emphasized the importance of biologically active compounds obtained from plants in their medicinal use (Bhattacharjee T. et al. 2020, Hedayat K.M. et al. 2020, Raji R.N. et al. 2019). Plant extracts are widely used in therapeutic applications, especially due to their antioxidant and antimicrobial properties. Current research has proven these effects on various plant species and revealed that secondary metabolites of plants play important roles in health (Srivastava J.P. et al. 1996, Wallace R.J. et al. 2004, Reghu R. Et al. 2017, Fazal H. et al. 2011)

Ginkgo biloba L. is one of the oldest tree species that has existed for millions of years and is considered a "living fossil" (Singh, B. et al. 2008). This plant, which has managed to remain structurally unchanged for more than 200 million years, contains various biologically active compounds and provides protection against insects, bacteria, and fungi thanks to its natural defense mechanisms. Ginkgo biloba leaves are rich in powerful antioxidant compounds such as kaempferol, quercetin, and isorhamnetin. These flavonoids help prevent diseases associated with oxidative stress by eliminating free radicals (Záhradníková L. et al. 2007, Ronowicz J. et al. 2013, Atzori C. et al. 1993).

Ginkgo biloba extracts have been found to have antiparasitic, antifungal, antibacterial, antiviral, and DNA-protective effects (Silva A.M. et al. 2019, Li M. et al. 2019, Perry E.K. 1999). Ginkgo extracts have been used for thousands of years in the treatment of bronchitis and other respiratory tract infections as well as cardiovascular diseases (Kleijnen, J. et al. 1992). In Western countries, it has been used in the treatment of atherosclerosis and cerebrovascular insufficiency since the 1960s (Xie L. et al. 2003. Diamond B.J.; et al. 2000). It has also been reported to be effective in the treatment of circulatory system disorders such as depression, memory loss, headache, and vertigo (Diamond, B.J.; et al. 2000). In this context, it is seen that *Ginkgo biloba* offers a wide therapeutic potential thanks to the bioactive compounds it contains and is an important plant in the field of health, especially due to its antioxidant and antimicrobial effects.

Response surface methodology (RSM) is an approach that includes mathematical and statistical techniques based on the fit of experimental data to a polynomial equation. RSM allows for numerical analysis of the behavior of a dataset and can be effectively applied in cases where multiple variables affect a response. The main objective of the method is to optimize these variables simultaneously to maximize the organizational output (Bezerra M.A. et al. 2008). Orthogonal experimental design and uniform design can only determine the best combination of variable ratios but cannot consider the optimal quality in the entire domain. RSM can predict the output (response) of a multivariate quadratic equation and this method has been successfully applied in various fields such as food (Y.Y. Chen. et al. 2012, S.H. Wu. et al. 2013] and medicine (M. B. Lan. Et al. 2012, Y.K. Hong. et al. 2013). The two main approaches frequently used in RSM are Box-Behnken Design (BBD) (Bezerra M.A. et al. 2008, J. Prakash et al. 2013) and Central Composite Design (CCD) (Bezerra M.A. et al. 2008, T. Zhu. et al. 2012). CCD can provide advantages such as orthogonality, rotation, and flexibility by varying the number of center points.

This study aimed to optimize antioxidant extraction conditions from *Ginkgo biloba* leaves by RSM method. Using five-level, three-factor CCD, the effects of temperature, time, and ethanol concentration on the extraction process were analyzed. The obtained results can be evaluated as a theoretical guide for the industrial production of antioxidants from natural sources.

MATERIALS AND METHODS

Ginkgo biloba leaves used in the experimental studies were obtained from a herbalist in Afyonkarahisar province of Turkey. The leaves were dried in a dark room for 15 days and then ground in a mill to make a fine powder.

Chemicals such as Folin-Ciocalteu reagent, gallic acid and quercetin standards, and aluminum chloride hexahydrate, methanol, and sodium carbonate were obtained from Sigma-Aldrich Co. (Istanbul, Turkey). Ultrapure water used in the experiments was produced with the Milli-Q System to have a conductivity of less than 0.05 μS cm^{-1}. All other chemicals used were of analytical purity.

Quantification analyses were performed using a dual-beam UV-visible spectrophotometer (Shimadzu UV1800, Japan) operating with 1.0 cm quartz cells and UV-probe software.

The ultrasound-assisted extraction process was carried out in a Wisebath brand ultrasonic water bath with a power of 900 W and a frequency of 50 kHz, equipped with a digital timer and a temperature control unit that provides both temperature and time control. Ultrasound waves were generated at a fixed frequency of 50 kHz at the bottom of the bath and spread into the water.

Ultrasound-Assisted Extraction

Ultrasound-assisted extraction is a method that enables the efficient extraction of plant components. In this study, the extraction process was carried out in a Bandelin Sonorex ultrasonic bath operating at a frequency of 50 kHz. For the process, 500 mg of dried and ground *Ginkgo biloba* leaves were added into a 100 mL volumetric flask, followed by the addition of 30 mL ethanol. The flask was then covered with aluminum foil and placed in an ultrasonic bath. The liquid level in the flask was adjusted to equal the water level in the ultrasonic bath. The aluminum foil helped to maintain the solution concentration by preventing the evaporation of ethanol. The device was operated following the experimental conditions specified in Table 1. After the extraction process was completed, the extracts were filtered through a 0.45-micron membrane filter. The extracts were stored in the refrigerator until total phenolic and total flavonoid analyses were performed.

Identification of Total Phenolic and Flavonoid Content

Total phenolic compounds were determined in each extract using the Folin-Ciocalteu method according to the previously described procedure (Petrović M. et al. 2022). For analysis, 1 mL of extract was mixed with 0.5 mL of Folin-Ciocalteu reagent, 2 mL of ultrapure water, and 4 mL of sodium carbonate solution (75 g L^{-1}). The resulting mixture was kept at 20 °C in the dark for 40 min and then the absorbance value was measured with a spectrophotometer at a wavelength of 765 nm. Gallic acid solutions prepared in methanol (five different concentrations in the range of 50-250 μg mL^{-1}) were used to create the calibration curve (Figure 1) and the results were expressed in gallic acid equivalents (GAE).

Total flavonoid content was determined using the Aluminum chloride colorimetric method (Lopes, J. D. et al. 2022). For analysis, 1.0 mL of extract, 4.0 mL of ultrapure water, and 0.3 mL of sodium nitrite solution (5%) were added to a 10 mL test tube. After waiting for five minutes, 0.3 mL of aluminum chloride solution (10%) was added. After six minutes, 4.0 mL of NaOH (4%) solution was added and then the total volume was completed to 10 mL

with ultrapure water. After the prepared solution was mixed well, the absorbance value against the blank solution was measured using a spectrophotometer (Shimadzu UV-1800, Japan) at a wavelength of 510 nm. Total flavonoid content was expressed as mg quercetin equivalent (QE) per 1 g of dried plant. The calibration curve was generated using quercetin solutions prepared in methanol (five different concentrations in the range of 50-250 µg mL^{-1}) (Figure 2) and the results were expressed as quercetin equivalents (QE).

Identification of Total Phenolic and Flavonoid Content

In order to determine the most suitable conditions for ultrasound-assisted extraction, the surface response methodology was applied. In this study, three independent variables were selected: extraction temperature, extraction time, and ethanol concentration. These factors were coded as X_1, X_2, and X_3, respectively, and were examined at five different levels (Table 1). The evaluation was made by taking the averages of the data obtained from 20 different experiments performed in three replicates. Accordingly, total phenolic and total flavonoid contents were determined. Each factor was coded at five different levels according to the equation given below (Prakash Maran, J. et al. 2013).

Table 1. Specific Variables and Rates of Central Composite Design.

Variables	Units	Symbols	Code levels				
			-1.68	-1	0	+1	+1.68
Ext. Temp.	°C	(X_1)	20	30	40	50	60
Ext. Time	min.	(X_2)	30	40	50	60	70
Ethanol Conc.	%	(X_3)	15	30	45	60	75

$$Y = \beta_0 \pm \sum_{j=1}^{k} \beta_j X_j \pm \sum_{j=1}^{k} \beta_{jj} X_j^{\,2} \pm \sum \sum_{i<j=2}^{k} \beta_{iJ} X_i X_J \qquad \text{Equation (1)}$$

In this equation, Y represents the dependent variable, x_i and x_j are independent variables (i and j vary from 1 to k). β_0 is the constant term, β_j is the linear coefficient, β_{ij} is the interaction coefficient and β_{jj} is the quadratic coefficient. Here, k indicates the number of independent variables, and k = 3 is determined within the scope of this study (G. E. P. Box. et al. 1957, Samavati V. 2013, Prakash Maran J. et al. 2013). The data obtained from the experiments were evaluated using the generalized least squares method and multiple regression analysis. Pareto analysis of variance (ANOVA) was applied to determine the statistical parameters. Surface response analysis was performed using Minitab 16 software, and the results were reported as mean ± standard error. $P < 0.05$ indicates statistically significant effects.

RESULTS AND DISCUSSION

Linearity of the Analytical Method

Absorbance values obtained against 5 different concentrations were plotted and calibration linearity was obtained. Five consecutive absorbance values were obtained for calibration of the device from 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm standard solutions.

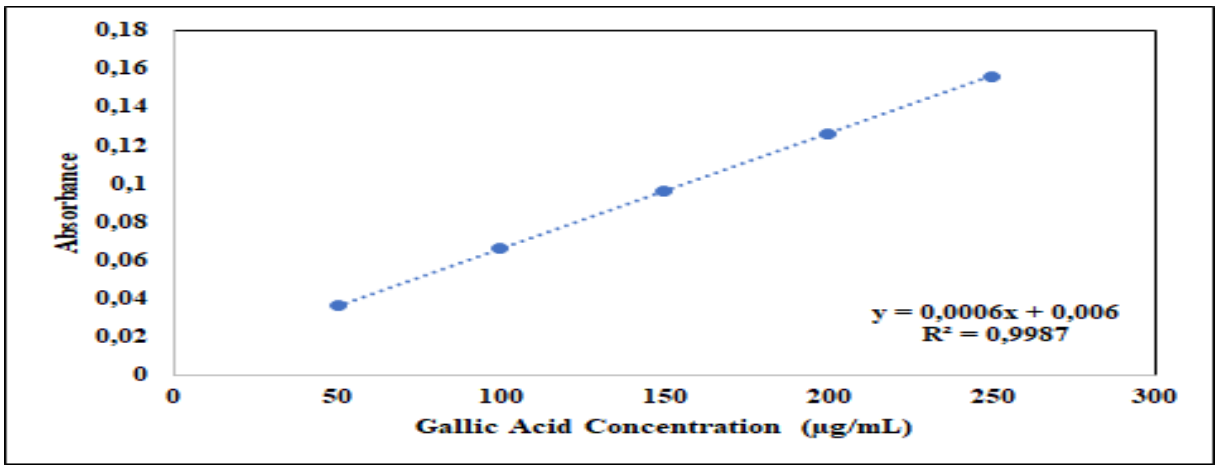

Figure 1. Linearity Plot Of Gallic Acid Standard Solutions at Different Concentrations.

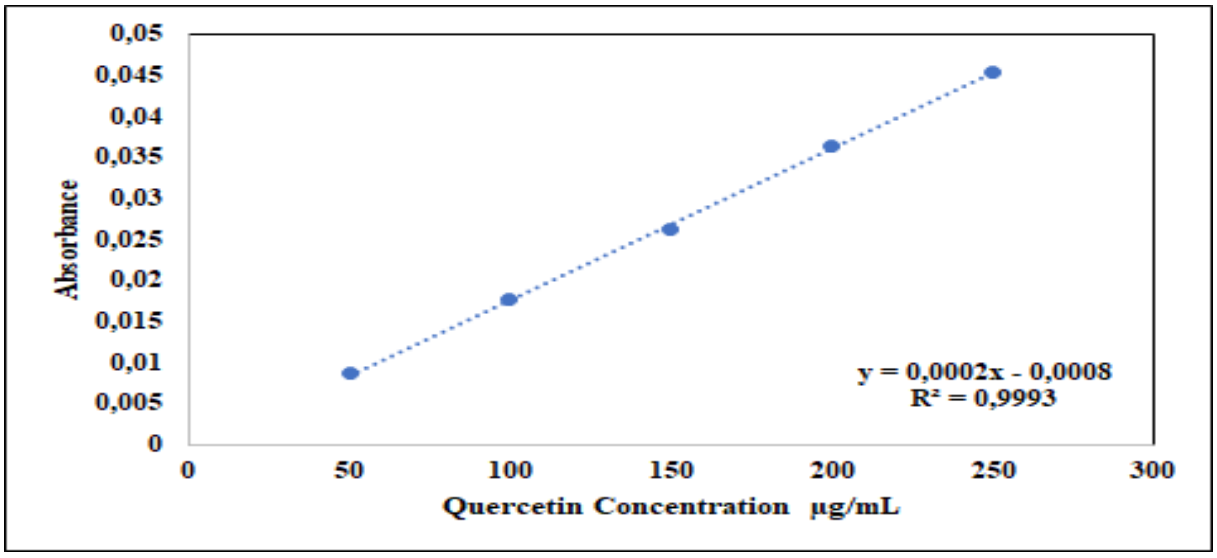

Figure 2. Linearity Plot of Quercetin Standard Solutions at Different Concentrations.

The central composite designs of the independent variables and the experimental results for total phenolic and total flavonoid contents are presented in Table 2.

Table 2. Central Composite Designs of İndependent Variables and Experimental Results for Total Phenolic and Total Flavonoid Contents.

Experimental study	Extraction temperature	Extraction time	Ethanol concentration	Total phenolic contents	Total flavonoid contents
	^{0}C	Minute	%	mg GAE g^{-1}	mg QE g^{-1}
1	30	30	45	35.00	6.16
2	40	40	30	41.67	7.49
3	30	50	45	60.94	8.50
4	50	50	45	52.78	8.29
5	40	60	30	51.87	10.51
6	30	50	45	57.49	9.30
7	30	50	45	61.70	8.58
8	10	50	45	47.66	6.97
9	20	40	30	33.69	6.68
10	40	40	60	50.33	8.06
11	30	50	15	41.23	10.16
12	40	60	60	58.79	11.34
13	30	70	45	53.52	10.93
14	30	50	75	60.96	7.61
15	20	60	60	61.62	8.39
16	30	50	45	62.26	10.73
17	20	40	60	45.16	9.38
18	30	50	45	63.47	11.79
19	30	50	45	64.59	12.01
20	20	60	30	50.55	9.38

Effect of Time on Extraction Efficiency

As shown in Table 1, it was observed that the extraction time at which the amount of phenolic and flavonoid substances was the highest increased continuously until the 45th minute, and after this time, the amount of total phenolic and total flavonoid substances decreased. The reason for this is that when the extraction time is raised, the cell walls of Ginkgo biloba leaves are completely separated and Ginkgo biloba leaves diffuse into the liquid

material. Overheating of Ginkgo biloba leaves during the long extraction time caused thermal degradation of the phenolic and flavonoid substance structure because phenolic and flavonoid molecules contain unstable chemical bonds such as unsaturated bonds, thus reducing the phenolic and flavonoid substance content. As a result, the preferable extraction time for phenolic and flavonoid extraction is 45 minutes. Response surface graphs showing the effect of extraction time and temperature on yield are shown in Figure 3.

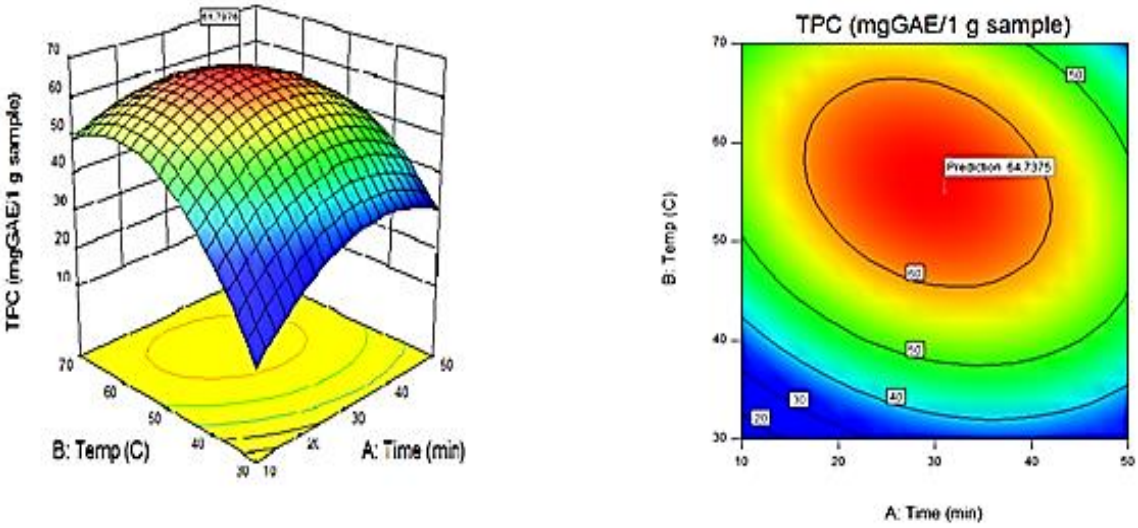

Figure 3. Surface Response Graphs Showing the Effect of Extraction Time and Temperature on Efficiency.

Effect of Temperature on Extraction Efficiency

Dried and ground Ginkgo biloba leaves were extracted at different temperatures. The amounts of total phenolic and flavonoid substances in the extracts were determined by UV-VIS spectrophotometer. The results showed that the amount of phenolic and flavonoid substances increased continuously until the extraction temperature of 50 ˚C and started to decrease after this point. High-temperature extractions improve extraction performance and mass transfer due to good desorption of solute from the active sites of the plant matrix. Initially, the increase in extraction efficiency with increasing temperature is because high temperature breaks phenolic and flavonoid substances from the plant cell and accelerates molecular movement. When the temperature increased above 50 ˚C, the extraction efficiency started to decrease. Temperatures greater than 50 ˚C cause the structure of phenolic and flavonoid substances to break down. Phenolic and flavonoid substances lose their activity and therefore the amount of phenolic and flavonoid substances decreases. As a result, the preferred temperature for phenolic and flavonoid extraction is 50 ˚C. Surface response graphs showing the effect of ethanol concentration and extraction temperature on yield are shown in Figure 4.

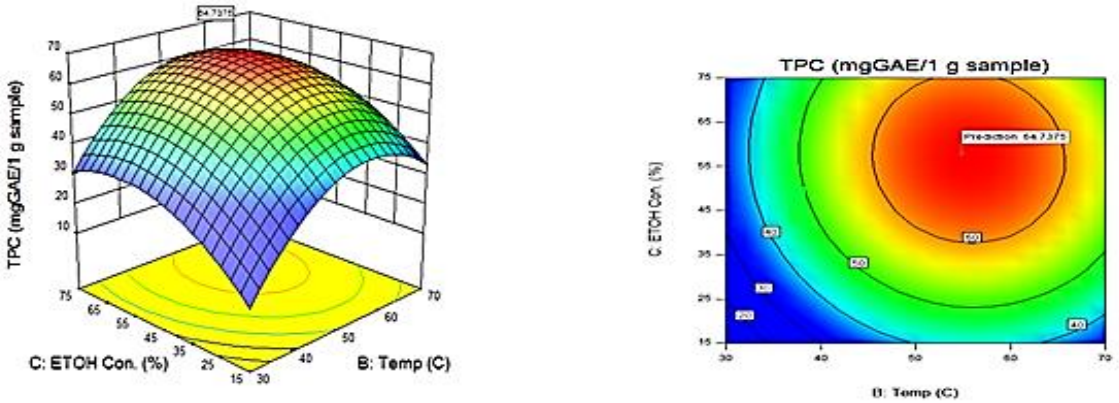

Figure 4. Surface Response Plots Showing The Effect of Ethanol Concentration and Extraction Temperature on Efficiency.

Effect of Ethanol Concentration on Extraction Efficiency

Ginkgo biloba leaf samples were extracted at different ethanol concentrations. Total phenolic and flavonoid content in the extracts were determined by Ultraviolet Visible Spectrophotometer. When the results were analyzed, the highest amount of phenolic and flavonoid substances was obtained at 75% ethanol concentration. As the

ethanol content increased up to 75%, an increase in total phenolic and flavonoid content was observed. As the ethanol concentration increased after this level, the amount of extracted substances decreased. Surface response graphs showing the effect of extraction time and ethanol concentration on the yield are shown in Figure 5.

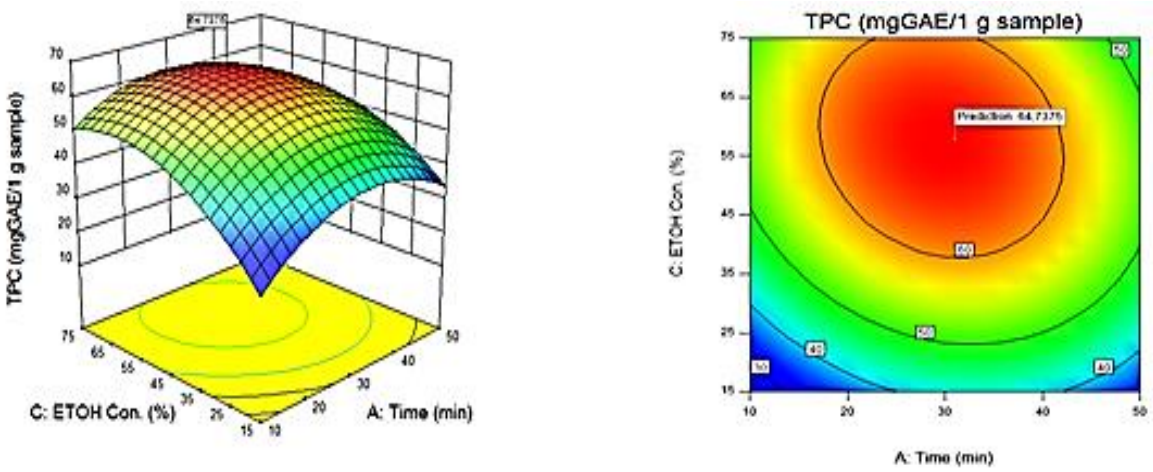

Figure 5. Response Surface Plots Show the Effect of Extraction Time and Ethanol Concentration on Efficiency.

Optimization of Ultrasonically Assisted Extraction by Surface Response Method

The single effects of the process parameters, known as the simultaneous single-factor approach, were implemented in the last section. This traditional model disregards the potential effects of interactions between the process parameters. The surface response methodology takes into account possible interactions. The table shows three parameters (Ethanol amount, time, and temperature) with minimum, medium, and maximum points.

20 different extraction methods were studied. The design was randomly selected by Expert software and the responses were recorded. Due to the software, a quadratic model using surface response methodology was obtained for the extraction yields, applying not only stepwise forward but also backward extinction regressions. A quadratic model using surface response methodology was derived from the software as given below. As a result of the analysis of the equation. 0.9569. The relationship between the predicted values and the experimental values is shown in Figures 6 and 7.

Model Fitting

The results of the ANOVA test for quadratic equations of the Design Expert 8.0.7.1 program are given in Equation 1 and Equation 2. The coefficients of the regression equation were calculated and the data were fitted in a second-order polynomial equation. The regression equation obtained from the ANOVA showed that the R2 (multiple correlation coefficient) was 0.9839. A value above 0.75 indicates that the model is appropriate. Regression analyses were performed at 95% confidence interval. The f value of the derived model is 41.46 and $p < 0.0001$ indicates that the derived model is appropriate.

This model calculated the overall variation in the data. Thus, this model was able to explain 98.39% of the variation in the response. R2 = 0.9584 and Predicted R2 = 0.9184. this shows that the model is good.

For a successful statistical model R2 value should be in the range 0-1.0. The adequate precision value of the current model is 28.388, which indicates that this model can be used for design. The adequate value is an indicator of the signal-to-noise ratio and values greater than 4 are essential preconditions for a model to be good. Simultaneously, relatively lower values of the coefficient of variation (CV = 3.13 %) indicate reliability and better precision of the values. The surface response method can be successfully applied to estimate the amount of flavonoids from the extraction of dried Ginkgo biloba leaves. Low values of the coefficient of variation indicate the reliability of the experimental results. In our study, the coefficient value (CV) is 3.13. The lower the coefficient of variation, the higher the reliability and sensitivity of the experimental results.In this framework, when the surface response graphs are analyzed; the possible conditions for the extraction of phenolic substances from Ginkgo biloba leaves; Time for extraction: 31.220, temperature: 54.119, Ethanol concentration: 57.944%. As a result, the optimum conditions of flavonoid extraction from Ginkgo biloba leaves: Time: 47.883 min., Temperature 36.336 ˚C, Ethanol concentration: 69.512%.

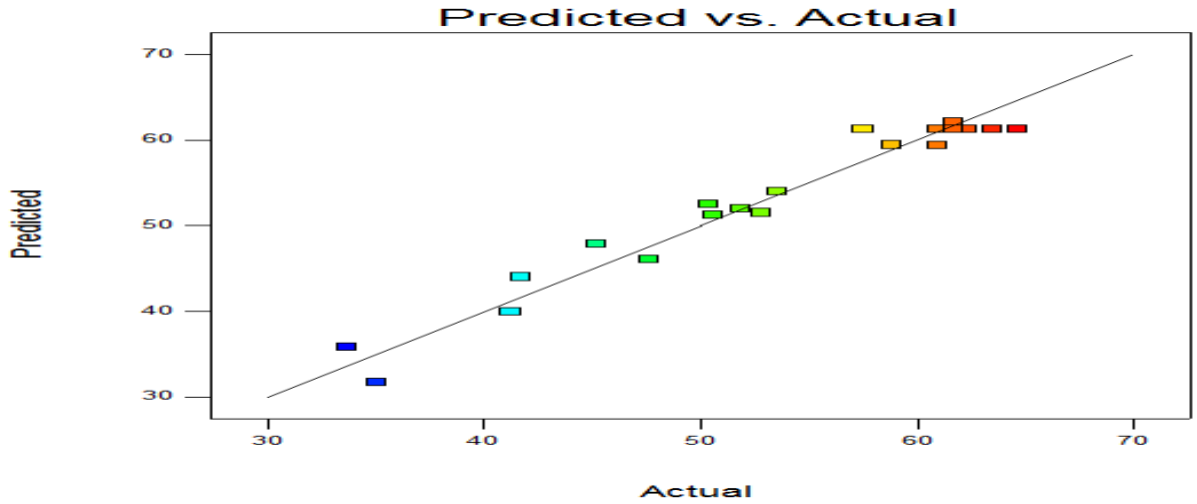

Figure 6. Linearity Plot of Experimental and Predicted Values of Total Phenolic Content in Ultrasonically Assisted Extraction.

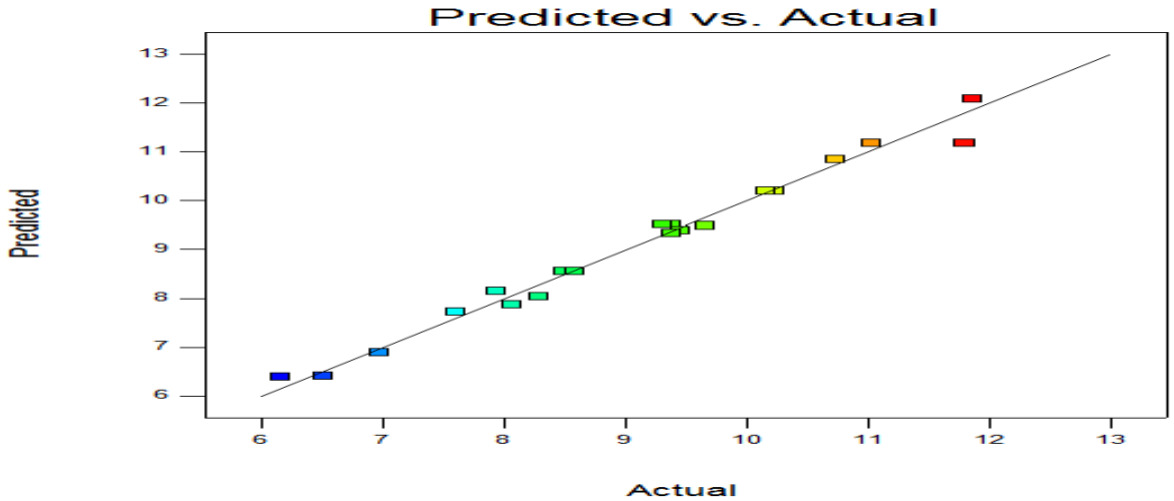

Figure 7. Linearity Plot of Experimental and Predicted Values of Total Flavanoid Content in Ultrasonically Assisted Extraction.

$TPC = -193.90977 + 3.18511 * X_1 + 5.79490 * X_2 + 1.74633 * X_3 - 0.031185 * {X_1}^2 - 0.0460085 * {X_2}^2 - 0.012888 * {X_3}^2$ (Equation 2)

$TFC = -8.61167 + 0.36503 * X_1 + 0.16116 * X_3 - 7.57172E - 004 * X_2 * -3.45813E - 003 * {X_1}^2 - 1.18677E - 003 * {X_3}^2$ (Equation 3)

X_1: Ethanol concentration. X_2: Time. X_3: Temperature

CONCLUSION

Response Surface Analysis Method was developed for the determination of total phenolic and total flavonoid content in the leaves of Ginkgo biloba, a member of the ginkgoinfa class, native to East Asia. Extraction temperature, extraction time, and solvent concentration were used as extraction parameters in the optimization. To find the optimal conditions where extraction temperature, ethanol concentration, and extraction time have a significant effect on the extraction results obtained by Ultrasonically Assisted Extraction, 20 different extractions were performed from Ginkgo biloba leaves at 3 different parameters (extraction time, extraction temperature, and ethanol concentration). Extraction parameters were optimized using Centrel Composite design in the Design Expert program. Extraction time (31.22 min), extraction temperature for total phenolics content: 54.11°C solvent concentration was 57.94% ethanol. The extraction time for total flavonoid content was 47.88 minutes, the extraction temperature was 39.33 °C, solvent concentration was 69.51% ethanol.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Declaration of Interests

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

The author read and approved the final manuscript. The author verifies that the Text, Figures, and Tables are original and that they have not been published before.

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