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Optimization Extraction of *Cladonia foliacea* (Huds.) Willd: Antioxidant Activity and Inhibition of the Key Enzymes Linked to Type II Diabetes

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ABSTRACT

Cladonia foliacea (CF) is a type of lichen belonging to the *Cladoniaceae* family, used as traditional medicine for various diseases. It is known that CF has strong antioxidant and antidiabetic effects originating from various secondary components. However, the bioactivity of CF is significantly affected by extraction conditions such as temperature, liquid/solid ratio, and solvent type/concentration. Herein, the extraction parameters (temperature, liquid/solid ratio, and methanol concentration) of CF were optimized by response surface methodology (RSM) based on maximum total phenolic content (TPC), antioxidant capacity, and α-glucosidase/α-amylase inhibitor activity. In the methanolic extraction of CF, 48.8 °C, 12.3 mL g^{-1} liquid/solid ratio, and 86.4% methanol concentration were determined as the optimum point. TPC, antioxidant capacity, α-glucosidase, and α-amylase inhibition activities of CF extracted under optimum conditions were determined as 5.55 mg GAE g^{-1}, 33.10 g sample/g DPPH, 68.78%, and 50.03%, respectively. These results suggest that extraction conditions may be a limiting factor in terms of bioactive properties and optimized extraction parameters may improve the potential antioxidant and inhibitory activity of key enzymes associated with type II diabetes of CF.

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Cladonia foliacea (Huds.) Willd'ın Ekstraksiyon Optimizasyonu: Antioksidan Aktivite ve Tip II Diyabet ile İlişkili Anahtar Enzimlerin İnhibisyonu

ÖZET

Cladonia foliacea (CF), çeşitli hastalıklar için geleneksel ilaç olarak kullanılan *Cladoniaceae* familyasına ait bir liken türüdür. CF'nin çeşitli sekonder bileşiklerden kaynaklanan güçlü antioksidan ve antidiyabetik etkilere sahip olduğu bilinmektedir. Ancak CF'nin biyoaktivitesi sıcaklık, sıvı/katı oranı ve solvent çeşidi/konsantrasyonu gibi ekstraksiyon şartlarından önemli ölçüde etkilenmektedir. Bu nedenle, bu çalışmada CF'nin ekstraksiyon parametreleri (sıcaklık, sıvı/katı oranı ve metanol konsantrasyonu) maksimum toplam fenolik madde miktarı (TFMM), antioksidan kapasitesi ve α-glukozidaz/α-amilaz inhibisyon aktivitesi baz alınarak yanıt yüzey yöntemi (YYY) ile optimize edilmiştir. CF'nin metanolik ekstraksiyonunda 48.8 °C, 12.3 mL g^{-1} sıvı/katı oranı ve %86.4 metanol konsantrasyonu optimum nokta olarak belirlenmiştir. Optimum koşullar altında ekstrakte edilen CF'nin TPC, antioksidan kapasitesi, α-glukosidaz ve α-amilaz inhibisyon aktiviteleri sırasıyla 5.55 mg GAE g^{-1}, 33.10 g örnek/g DPPH, %68.78 ve %50.03 olarak belirlenmiştir. Bu sonuçlar, ekstraksiyon koşullarının biyoaktif özellikler açısından sınırlayıcı bir faktör olabileceğini ve optimize edilmiş ekstraksiyon parametrelerinin CF'nin potansiyel antioksidan ve tip II diyabetle bağlantılı anahtar enzimlerin inhibisyon aktivite etkisinin iyileştirebileceğini düşündürmektedir.

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Optimizasyon

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder caused by an uncontrolled increase in sugar in blood plasma. Although there are different types of diabetes, type I and type II are common in the social. Type I diabetes is generally characterized by damage to the ß-cells involved in insulin secretion, whereas type II diabetes occurs with irregular insulin secretion and resistance (Prathapan et al., 2012). Type II diabetes affects approximately 90% of diabetes patients worldwide (Bhutkar and Bhise, 2012). Along with many factors are involved in its occurrence, it has also been linked to increased oxidative stress in the body in recent years (Găman et al., 2020). Oxidative stress, which occurs when free radicals formed during metabolic activity cannot be destroyed or inactivated by antioxidants, cause important functional disorders such as proteins, lipids and nucleic acid damages. (Vincent et al., 2004). While insulin supplementation by injection is required in type I diabetes, the glucose level in plasma can be lowered with therapeutic drugs or insulin sensitizers in type II diabetes. Therefore, considering the side effects of synthetic antioxidants (Zhang et al., 2012) and medicinal drugs for diabetic (Cariou et al., 2012), products with natural and herbal ingredients are accepted as an up-to-date approach in the treatment of type II diabetes and damage caused by free radical-induced oxidative stress (Page and Reisman, 2013). Another approach is the inhibition of α-glucosidase and α-amylase enzymes in the small intestine that convert complex carbohydrates into absorbable form (Hsieh et al., 2010; Reddy et al., 2010).

Lichens are a life form in which algae and fungi are in a symbiotic relationship and have been used by people as a source of healing in folk medicine for a long time (Huneck, 1999). Lichens have a variety of secondary metabolites, and most of these have been concluded to be of fungal origin that is part of the lichen (Shrestha and Clair, 2013). These secondary metabolites exhibit remarkable biological activity such as antimicrobial (Yılmaz et al., 2004), antioxidant (Kosanić et al., 2014), antitumor (Emsen et al., 2016), anti-inflammatory (Joshi et al., 2020), enzyme inhibitor (Hengameh et al., 2016), cardiovascular protective (Behera et al., 2012), antidiabetic (Thadhani and Karunaratne, 2017), and gastrointestinal protective (Nayaka and Haridas, 2020). *Cladonia* genus has more than 500 species worldwide and contains high amounts of secondary metabolites (Ahti, 2000). These secondary metabolites, such as zeorin, methyl-β-orcinol carboxylate, methylorsellinate, and usnic acid of *Cladonia* sp., are known to have antioxidant and antidiabetic potential (Verma et al., 2012; Karunaratne et al., 2014; Thadhani and Karunaratne, 2017; Cavalloro et al., 2021). Besides, polyphenols, which are secondary metabolites, can be used to treat type II diabetes due to their properties of protecting pancreatic ß-cells and their α-amylase, α-glucosidase inhibition properties (Xiao and Hogger, 2015). *Cladonia foliacea* (Huds.) Willd (CF) exhibits high amounts of usnic acid, fumarprotocetraric acid (Litterski and Ahti, 2004), and atranorin compounds (Yılmaz et al., 2004). Fumarprotocetraric acid, one of these compounds, exhibits antioxidant activity (Kosanić et al., 2014), usnic acid and atranorin compounds show both antioxidant and antidiabetic activity (Thadhani and Karunaratne, 2017; Ahamed et al., 2019; Hoa et al., 2020).

Extraction conditions are fundamental to make maximum use of secondary metabolites that lichens contain because extraction depends on many factors such as extraction method, particle size, solvent type and concentration, temperature, and time (Dai and Mumper, 2010). Response surface methodology (RSM) is an optimization of stochastic models that successfully applies the effects of factors on the answers by minimizing the trial points in a process and using mathematical and statistical techniques (Myers et al., 2016).

In general, there are studies in the literature that reveal antioxidants (Zambare and Christopher, 2012; Fernández-Moriano et al., 2016) and their antidiabetic activity (Shivanna et al., 2015; Thadhani and Karunaratne, 2017) for some lichen. Although recent studies on the bioactive properties of lichens have gained momentum, their therapeutic effects have not been adequately explained. This study was aimed to optimize the extraction conditions of *Cladonia foliacea* (Huds.) Willd for maximizing in-vitro antioxidant and antidiabetic properties.

MATERIALS and METHODS

Collection and identification of the lichen

Collection address of specimen: Turkey, Trabzon, Maçka, Vicinity of Sümela Monastery, Coşandere position, Q*uercus* sp. communities, calcareous rocks, Date: 19.08.2016, 40º45'560" N, 39º36'718"E, 570 m [MK-4487].

Identification: The specimen was examined with an Olympus SZX10 stereomicroscope. Specimen is deposited in the Yozgat Bozok University Herbarium. *Cladonia foliacea* (Huds.) Willd has squamulose to foliose thallus, squamules 0.6–4 cm long, yellowish, olive green, irregularly lobed, usually white unbranched rhizines at the edges. It has a very wide distribution area in Turkey.

Preparation of the lichen extract

The lichen sample was dried under room conditions. Then, the dried sample was grounded (Waring 8011 ES blender, USA). Preparation of the extracts was performed according to the method of Doğan et al. (2020) with some modifications. 1 g of ground lichen sample was extracted with methanol in a shaking water bath (Wisd, Korea) for 120 minutes at a specific concentration, temperature, and liquid/solid ratio according to the experimental design.

Experimental design and optimization

Box-Behnken experimental design (BBD) type applied in RSM (Design Expert 11.0.0 software) was chosen to optimize extraction conditions. Considering into account the TPC, DPPH (IC_{50}), α-glucosidase, and α-amylase activities of the samples (responses), the extracts made according to the independent variables temperature, liquid/solid ratio, and methanol concentration were optimized. Experimental points were formed by considering three levels of independent variables shown in Table 1.

Table 1. Levels of independent variables
Çizelge1. Bağımsız değişkenlerin seviyeleri

Independent variables *Deneme noktaları*	**Units** *Birimi*	**Symbol** *Sembol*	**Coded levels** *Kodlanmış seviyeler*		
			-1	0	1
Temperature *(Sıcaklık)*	°C	X_1	30	45	60
Liquid/solid ratio (*Sıvı/katı oranı*)	mL g^{-1}	X_2	10	20	30
Methanol concentration *(Metanol konsantrasyonu)*	%	X_3	50	75	100

The effect of independent variables such as temperature, liquid/solid ratio, and methanol concentration on responses TPC, IC_{50}, α-glucosidase, and α-amylase activities of the samples was determined using a quadratic polynomial regression equation (Equation1.).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where Y is the predicted response; β_0 the constant, β_1, β_2, β_3 are the linear coefficients; β_{11}, β_{22}, β_{33} the interaction coefficients; β_{12}, β_{13}, β_{23}; quadratic coefficients; X_1, X_2, X_3 the independent variables.

Total phenolic content (TPC)

To determine TPC of extracts; 0.4 mL sample, 2 mL diluted (10 times) Folin-Ciocalteu (FC) with 1.6 mL 7.5% Na_2CO_3 were mixed and vortexed. At the end of 60 minutes of incubation at room ambient in a dark place and its absorbance was recorded at 765 nm by a spectrophotometer (Shimadzu UV-1700, Kyoto, Japan). The results were given based on the gallic acid equivalent (GAE) (Singleton et al., 1999).

Antioxidant capacity assay

The antioxidant capacity of the samples was tested by considering the DPPH method. For DPPH analysis, 0.1 mL diluted extract was mixed with the methanolic DPPH solution prepared at a concentration of 25 mg L^{-1} and kept at room ambient for 30 minutes. After that, the absorbance of the extract was recorded at 515 nm (Brand-Williams et al., 1995) by a spectrophotometer (Shimadzu UV-1700). The results of the analysis were shown as IC_{50} (g sample/g DPPH).

Enzyme inhibitor activity assay

In order to determine the α-amylase inhibition activity, 1 mL extract was mixed with 1 mL potato starch solution and 1 mL 20 mM NaH_2PO_4 and then kept at 37 °C for 5 minutes. After adding 1 mL α - amylase solution to the mixture to initiate the enzymatic reaction, 30 minutes later, 0.5 mL 5.31 M Rochella Salt and 0.5 mL 96 mM $C_7H_4N_2O_7$ (3,5-dinitrosalicylic acid) solution were mixed. To terminate the chemical reaction, the mixture was held at 100 ° C for 15 minutes, and then the absorbance was recorded at 540 nm by a spectrophotometer (Shimadzu UV-1700) (McDougall et al., 2005). To determine α-glucosidase inhibition activity, 50 µL extract, 1250 µL 67 mM KH_2PO_4, and 50 µL α-glucosidase mixture were incubated at 37 °C for 5 minutes. 125 µL 10 mM p-Nitrophenyl-ß-D-glucopyranoside solution was added and the enzymatic reaction started. About 20 minutes later, 2 mL 100 mM Na_2CO_3 was mixed to terminate the reaction, and were recorded the absorbances at 400 nm by a spectrophotometer (Shimadzu UV-1700) (Cam et al., 2020). The inhibition activity of the

samples was calculated by considering the formula below.

$$\textbf{\textit{Antidiabetic activity}}\ (\%) = \frac{ABS_{control} - ABS_{sample}}{ABS_{control}} x100 \quad (2)$$

$ABS_{control}$ and ABS_{sample} represent the absorbances of control and samples, respectively.

Statistical analysis

Superimposed 3D surface plots were created by Mathematica program package 7 (Wolfram Research). Besides, to find the significance of the data's differences, the SPSS 22.0 software program (SPSS Inc., Chicago, IL) was used.

RESULTS and DISCUSSION

Comparison experimental-predicted values and checking of model fitting

The predicted and experimental results of all responses, which are according to the Box-Behnken design experiment points, were given in Table 2. The difference between the experimental and predicted values of the experimental design points' responses is less than 0.05, indicating a reliable model.

Table 2. Box Behnken design with experimental values and predicted data for the independent variables
Çizelge 2. Box Behnken deneme dizaynına göre deneysel değerler ve tahminlenmiş veriler

Run *Den. nok.*	Independent variables *Bağımsız değişkenler*			Responses *Yanıtlar*							
	Temperature Sıcaklık (°C)	Liquid/solid ratio Sıvı/katı oranı (mL g^{-1})	Methanol concentration Metanol konsantrasyonu (%)	TPC (mg GAE g^{-1})		IC_{50} (gsample/gDPPH)		α-glucosidase (%)		α-amylase (%)	
				Pre. *Tah.*	Exp. *Den.*	Pre. *Tah.*	Exp. *Den.*	Pre. *Tah.*	Exp. *Den.*	Pre. *Tah.*	Exp. *Den.*
1	45	10	100	5.07	5.10	31.56	35.11	64.86	62.00	44.27	43.10
2	30	20	50	2.78	2.76	111.76	109.91	26.05	21.67	11.36	10.78
3	45	30	100	3.91	3.99	69.80	65.56	51.72	50.00	34.59	33.00
4	60	20	50	3.29	3.42	102.87	104.03	35.56	35.36	23.00	20.82
5	45	20	75	5.11	5.20	48.28	60.1	64.07	66.96	45.65	45.21
6	45	20	75	5.11	5.16	48.28	41.07	64.07	65.06	45.65	46.66
7	45	10	50	4.66	4.58	88.08	92.32	44.69	46.41	41.42	43.00
8	45	30	50	3.13	3.10	104.29	100.74	34.77	37.62	28.83	30.00
9	30	20	100	3.59	3.46	69.63	68.47	44.88	45.07	18.87	21.05
10	30	10	75	4.85	4.95	61.38	58.99	60.00	62.66	26.81	25.80
11	60	30	75	3.81	3.71	76.36	78.75	57.71	55.05	24.10	25.11
12	30	30	75	3.10	3.15	83.01	88.41	39.22	40.74	21.00	20.41
13	45	20	75	5.11	4.98	48.28	43.66	64.07	60.19	45.65	45.07
14	60	20	100	3.68	3.70	54.01	55.86	53.85	58.23	24.08	24.66
15	60	10	75	4.74	4.69	43.52	38.12	59.99	58.46	40.56	41.15

Exp.: Experimental value, Pre.; Predicted data
Den: Deneysel değer, Tah.: Tahminlenen veri, Den. nok.: Deneme noktası

For the reliability of the 2nd-order polynomial equations derived from the model; Statistical parameters such as regression (p-value), coefficient of determination (R^2), adjusted R^2 (R^2_{adj}), estimated R^2 (R^2_{pred}), and lack of fit were evaluated. The lack of fit F-values of 2.02, 0.46, 1.13, and 7.14 imply the lack of fit is not significant relative to the pure error for the TPC, IC_{50}, α-glucosidase, and α -amylase of the samples, respectively. R^2 values of the responses were determined as 0.981, 0.937, 0.955, and 0.981 for TPC, IC_{50}, α-glucosidase, and α–amylase, respectively (Table 3).

Moreover, the difference between R^2_{pred} and R^2_{adj} is less than 0.2. If the difference between Adj-R^2 and pre-R^2 values is less than 0.2 and the R^2 and R^2_{adj} values are above 90%, the model is suitable (Myers et al., 2004). To increase the effectiveness of the model on the responses, unimportant independent variables ($p > 0.05$) were modified by removing them from the 2nd-order polynomial equation. This situation is critical in determining important factors' effect on responses (Fernández-Martínez et al., 2011).

Effect of the extraction conditions on TPC and antioxidant activity

The study results show that the TPC varies between 2.76 and 5.20 mg GAE g^{-1} (Table 2). The effect of all independent variables, the extraction conditions, on the TPC was significant ($p<0.05$). The highest TPC value was determined at the midpoints of the extraction conditions. As shown in Figure 1., the TPC value increased as the temperature, liquid/solid ratio, and methanol concentration increased up to 45 °C, 20 mL g^{-1}, and 75%, respectively, and then decreased effectively. Temperature is an essential factor in the extraction process due to its effects, such as softening the tissues, increasing the solubility, and decreasing the surface tension in the transition of phenolics to the solvent.

Table 3. 2nd-order polynomial equations and statistical parameters for model fitting of responses
Çizelge 3. 2. dereceden polinomial denklemler ve yanıtların model uyumluluğu için istatistiksel parametreler

Responses *Yanıtlar*	2nd-order polynomial equations *2. dereceden polinomial denklemler*	Regression (p-value)	R^2	R^2_{adj}	R^2_{pred}	Lack of fit (p-value) *Model uyumsuzluğu (p-value)*	Lack of fit (F-value) *Model uyumsuzluğu (F-value)*
TPC	$5.07+0.15X_1-0.671X_2+0.299X_3+0.205X_1X_2-0.918X_1^2-0.850X_3^2$	<0.0001	0.981	0.967	0.924	0.367	2.02
IC_{50}	$50.32-6.13X_1+13.61X_2-22.75X_3+14.21X_1^2+21.57\ X_3^2$	<0.0001	0.937	0.901	0.835	0.814	0.46
α-glucosidase	$63.79+4.62X_1-5.77X_2+9.28X_3+4.63X_1X_2-9.35X_1^2-14.57X_3^2$	<0.0001	0.955	0.921	0.811	0.539	1.13
α-amylase	$45.78+4.21X_1-5.57X_2+2.15X_3-2.66X_1X_2-17.75X_1^2-8.60X_3^2$	<0.0001	0.981	0.967	0.930	0.128	7.14

On the other hand, it is a known fact that high-temperature damages phenolic compounds (Dent et al., 2013). For this reason, temperature is one of the most critical factors in extraction. For this purpose, it is vital to optimizing the extraction temperature specific to the product. The transition of phenolic compounds to the solvent is highly related to the solvent type. The highest TPC extraction was detected in a mixture of 75% methanol and 25% water. In general, it is known that the use of a binary solvent system (mixed solvent) as a solvent can provide more effective extraction performance than using a single solvent (Markom et al., 2007). In the literature, it has been emphasized that generally, single-type organic solvents have a higher effect on the extraction of lichens' phenolic compounds (Karthik et al., 2011; Mitrović et al., 2011; Mendili et al., 2021). However, lichens contain complex compounds (Shukla et al., 2010), and there is limited information about their solubility in different solvents such as water or binary solvent system. A considerable amount of TPC was detected in the aqueous extracts of 24 lichens, including the *Cladonia* genus (Zagoskina et al., 2013).

DPPH is a widely used method to determine the antioxidant activity of extracts rapidly or pure compounds. The lowest IC_{50} for DPPH value was determined as at the extraction point at 45 °C with 10:1 liquid/solid ratio and 100% methanol (Table 2). Most of the phenolics have an antioxidant effect, which is expressed by the IC_{50}. Decreasing the IC_{50} value also means that the amount of sample required scavenging half of the DPPH radical decreases. A negative correlation was found between IC_{50} and TPC, which can be expressed with this phenomenon, as shown in Figure 2. Although the individual yield of phenolics can be high in extractions with binary-solvent, extracts using 100% methanol with the highest antioxidant activity were also found in this study. This may be since CF's phenolic components with a high antioxidant effect dissolve better in methanol, which has lower polarity than water. In short, mono-solvent systems can be more efficient in extracting the components with high antioxidant activity of CF. The antioxidant activity results obtained from the study are supported too by studies in the literature that some lichen extracts or compounds isolated solely with methanol from lichen have higher antioxidant activities (Odabasoglu et al., 2004; Khadhri et al., 2019). Similar to the result Anar et al. (2016) and Khadhri et al. (2019) reported that the methanolic extraction of CF contains high antioxidant activity and did not show any mutagenicity effect.

Effect of the extraction conditions on the α -amylase and α-glucosidase

In Figure 2, the effect of independent variables on α-amylase and α-glucosidase is shown in the perturbation plot. While the perturbation plot shows the effect of all independent variables on the responses together, it also gives information about a variable's effect. In contrast, other variables are kept constant at the central point. It is clear from the perturbation plot that the effect of α-glucosidase and α-amylase decreases as the liquid/solid ratio increases for both. The liquid/solid ratio had the reverse effect between the temperature and methanol concentration had been detected. The study results show that the α -amylase and α-glucosidase inhibition effect vary between 10.78-46.66% and 21.67-66.96%, respectively (Table 2). The inhibition effect of α -amylase, and α-glucosidase was also the highest at the middle points of the coded level (0-0-0), like TPC. This circumstance can be explained by the inhibition of the enzyme α-amylase and α-glucosidase, which play a role in carbohydrate degradation by phenolic compounds (McDougall et al., 2005). Pancreatic α-amylase and α-glucosidase are the main enzymes in the gastrointestinal system that convert complex

carbohydrates into simple sugars. Inhibition of these enzymes is a therapeutic approach applied in the control of Type II diabetes. Acarbose, miglitol, and voglibose are used as active drugs in the treatment of Type II diabetes. These clinical applications prevent postprandial hyperglycemia by inhibiting α-glucosidase and α-amylase enzymes or restricting monosaccharides' absorption (Dash et al., 2018). However, there are side effects associated with using these drugs (Su et al., 2013). For such reasons, the use of naturally derived and to investigate new agents with antidiabetic properties becomes important. The antidiabetic effect of some *Cladonia* sp. was reported by Valadbeigi and Shaddel (2016) and Zhang et al. (2012) for *Cladonia rei* and *Cladonia humilis*, respectively. Also, It is known that usnic acid Verma et al. (2012) and atranorin Ahamed et al. (2019) compounds, which are one of the main biotechnological components of the CF and isolated from various lichens, have antidiabetic effects. However, no studies have been found to determine the α-glucosidase and α-amylase inhibition effect of CF and further optimize the extraction factors on antidiabetic activity. The results showed that the extract of CF was the strongest antidiabetic activity. Although the independent variables showed the same effect in the extraction process, the inhibition effect of α-glucosidase was higher than the inhibition effect of α-amylase.

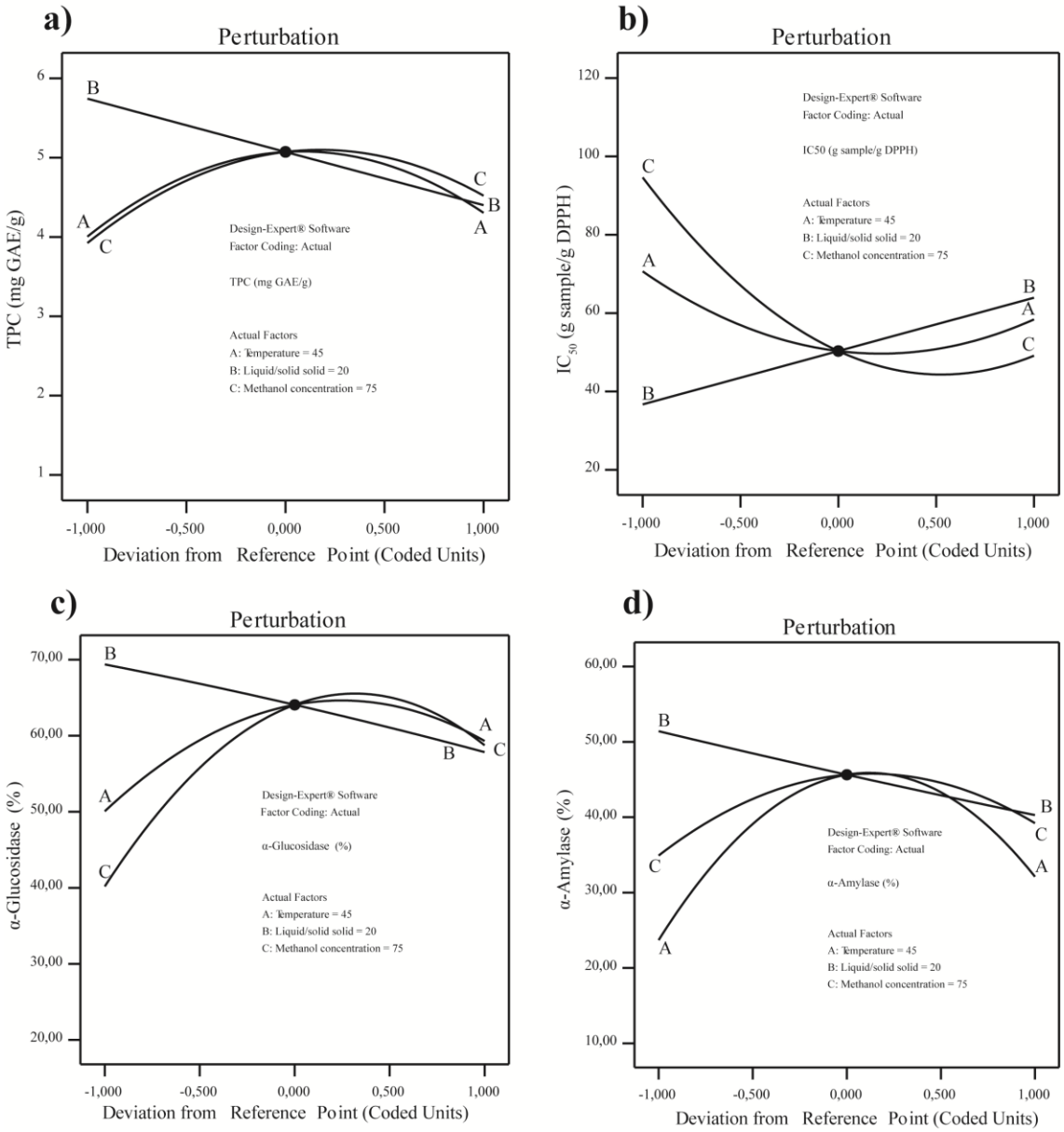

Figure 1. Perturbation plots showing the effect of factors on the (a) TPC, (b) IC_{50} , (c) α-Glucosidase, and (d) α-Amylase

Şekil 1. Faktörlerin (a) TPC, (b) IC_{50}, (c) α-Glukozidaz ve (d) α-Amilaz üzerindeki etkisini gösteren pertürbasyon grafikleri

Optimization and model validation

The desired criteria of responses were chosen to determine the best extraction conditions. Accordingly, while the antidiabetic activity and TPC were maximized, the lowest IC_{50} value reflecting a high antioxidant effect was assigned. No restrictions were done in process conditions such as temperature, liquid/solid ratio, and methanol concentration. The optimum point was determined based on the highest desirability score. Optimum conditions predicted data of the models, and triplicate experimental values at optimum points are given in Table 4. The difference between these values is less than 5%, which indicates the reliability of the model.

It was clearly seen in Figure 2., the points with the highest values of the amount of TPC, α-amylase, and α-glucosidase inhibition effect were so close to each other, while they are quite distant with IC_{50}. When considered individually, it is seen that although the maximum values for all responses are reached under different extraction conditions, they are different from the optimum points. However, this situation is quite normal, and factors should be evaluated as a part of a whole rather than affecting the responses one by one. This is one of the aims of optimization. The fact that predicted data derived from the mathematical model with the participation of four responses and the experimental value analyzed at optimum points is higher than the value made by individual extraction already summarizes the situation. Studies suggest that TPC is associated with α-amylase and α-glucosidase McDougall et al. (2005) and that oxidative stress caused by free radicals increases Type II diabetes (Page and Reisman, 2013). Considering all of these, it is vital to evaluate a substance and pure compound as a whole active component in the optimization process.

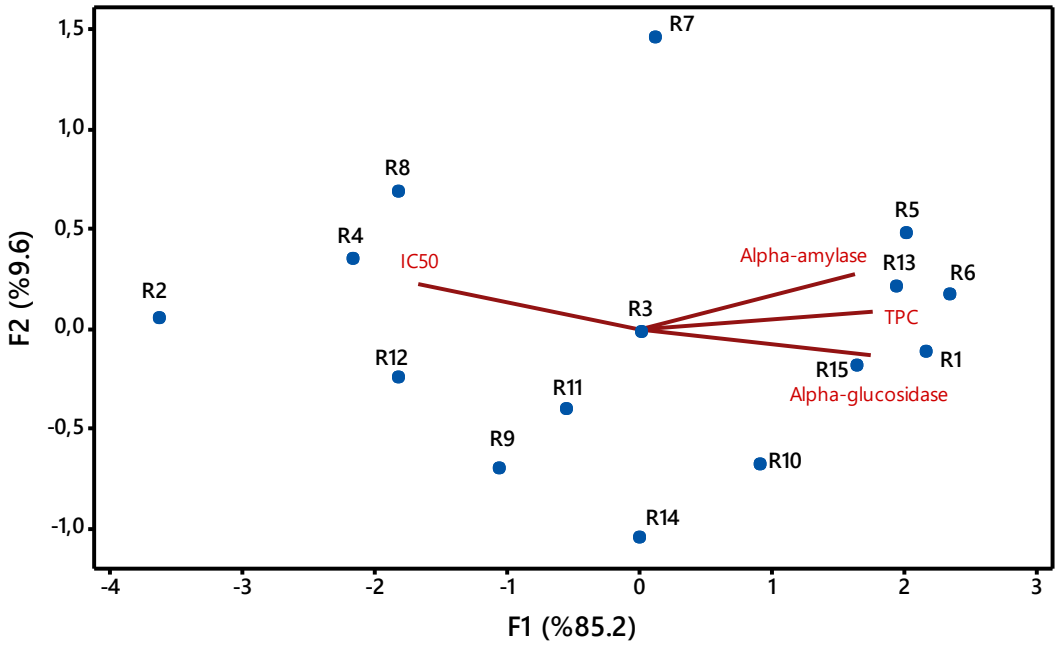

Figure 2. Principal component analysis (PCA) bi-plot containing experimental points and responses
Şekil 2. Deneysel noktaları ve yanıtları içeren bi-plot temel bileşen analizi (PCA)

Table 4. Optimum points and experimental value-predicted data at these points
Çizelge 4. Optimum noktalar ve bu noktalardaki deneysel değerler-tahminlenen veriler

Optimum points *(Optimum noktalar)*							
Temperature (°C) *Sıcaklık*	Liquid/solid ratio (mL g^{-1}) *Sıvı/katı oranı*	Methanol conc. (%) *Metanol kon.*	Desirability score *Arzu edilirlik*	Response *Yanıtlar*	Pre. data *Tah. değ.*	Exp.value *Den.veriler*	Differences(%) *Fark*
48.8	12.3	86.4	1.00	TPC (mg GAE g^{-1})	5.49	5.55±0.63	1.08
				IC_{50} (g sample/g DPPH)	33.36	33.10±0.15	0.78
				α-glucosidase (%)	69.09	68.78±0.77	0.45
				α-amylase (%)	49.69	50.03±0,36	0.68

Methanol conc.: Methanol concentration, Pre. data: Predicted data, Exp.value: Experimental value
Metanol kon.: Metanol konsantrasyonu, Tah. değ.: Tahminlenen değerler, Den.veriler: Deneysel veriler

CONCLUSION

In conclusion, CF has exhibited the highest potent TPC, antidiabetic and antioxidant activity at 48.8 °C with a 12.3 liquid/solid ratio and 86.4% methanol concentration. TPC, IC_{50}, α-glucosidase, and α-amylase activities performed at optimum norm were determined as 5.55 mg GAE g^{-1}, 33.10 g sample/g DPPH, 68.78%, and 50.03%, respectively. Lichens have been used as various folk medicine remedies for many years, and their proven pharmaceutical effects. On the basis present study, it could be declerated that CF is a good source of antioxidants and antidiabetic. Moreover, it can be used as a versatile natural source to enhance many different foods or guide medicinal purposes.

Researchers Contribution Rate Declaration Summary

The authors declare that they have contributed equally to the article.

Conflicts of Interest Statement

The article authors declare that there is no conflict of interest between them.

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