Process Optimization of Microwave-Assisted Extraction of Phenols and Flavonoids and Radical-Scavenging Capacity of Microalga *Spirulina platensis* by Response Surface Methodology

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Abstract
Pharmaceutical, nutritional and food industries have recently become interested in the potential of *Spirulina platensis* as a natural antioxidants. Phenolics are important compounds that are known for their antioxidant bioactivities and other health-promoting properties. In this study, response surface methodology (RSM) was used to optimize the conditions for the extraction of total phenolic content (TPC), total flavonoid content (TFC) and determine the free radical scavenging activity ($IC_{٥٠}$ value) from *S.platensis* extract by microwave at constant temperature. The investigated factors in this method were the solvent ratio (ethanol/water at ٠ to ١٠٠٪) and extraction time (٦٠-١٢٠ sec). Experiments were carried out based on a central composite design. The results showed that the extraction conditions had a significant effect on the $IC_{٥٠}$ value, TPC and TFC extraction. The optimal conditions for TPC (٢.٢٨ mgGAE/g) and TFC (٠/٧٥ mg RE/g) were determined to be ethanol content (١٠٠ % v/v) - ٦٠ sec and water content (١٠٠ % v/v) - ٦٠ sec respectively. The optimal conditions for $IC_{٥٠}$ value (١,٠٤ mg/g) were determined to be ethanol content (١٠٠ % v/v) - ٦٠ sec. These optimized conditions allow the rapid and maximum extraction of bioactive compounds from *S.platensis*.

Keywords: Flavonoids, Microwave, Phenols, Response Surface Methodology, *Spirulina platensis.*

Introduction

Recently, much attention has been focused on the microalgae as sources of novel, biologically active compounds such as phycobiline, phenols, terpenoids, steroids and polysaccharide (Abd El Baky et al., ۲۰۰۹). Algal phenolic compounds were reported to be a potential candidate to combat free radicals (Estrada et al., ۲۰۰۱). Spirulina, a filamentous blue-green cyanobacterium, botanists classify it as a micro alga belonging to cyanophyceae, it has a simple structure but a complex composition which growing in alkaline water bodies (Ravi et al., ۲۰۱۰). Due to its rich chemical composition and high nutritional value, it has been recognized by the World Health Organization as a superfood (Shao et al., ۲۰۱۹).

Spirulina is a good source of essential amino acids, vitamins, carbohydrates, macro- and trace minerals, and other nutrients. The composition percent dry weight of spirulina is ٦٤–٧٣٪ protein (٦٠-٧٠٪ by dry weight), ١٢–١٧٪ carbohydrate, ٥–٧٪ lipids, ٠/٩٪ P, and ١٠/٣–١١/٦٪ N, vitamins, minerals, essential fatty acids and other nutrients (Deng and Chow, ۲۰۱۰). Spirulina and its components have been shown to have positive benefits across a range of human health indications from malnutrition to antioxidant properties (Abu Zaid et al., ۲۰۱٥). Among large number of *Spirulina* species, three species of *Spirulina*, including *Spirulina platensis* (*Arthrospira platensis*), *Spirulina maxima* (*Arthrospira maxima*) and *Spirulina fusiformis* (*Arthrospira fusiformis*) are most intensively investigated as those *Spirulina* species are edible with high nutritional as well as potential therapeutic values (Deng and Chow, ۲۰۱۰). Antioxidant can slows or prevents the oxidative or damage from oxygen process caused by free radicals and can enhance the biological functions of cells by virtue of their radical scavenging activities (Jayaprakasha et al.,۲۰۰۸). Since antioxidants are vital for their role to delay or inhibit oxidation of cellular components, adequate intake of these compounds in the diet will be beneficial to protect against oxidative damages to the cell (Wang et al., ۲۰۰۹). In this regards, extracts of many medicinal plants and microalgae rich in phenolic compounds are increasingly used either as additive in food or consumed directly as a natural source of antioxidant (Wong et al., ۲۰۰٦).

Classical organic solvent extraction techniques, including maceration (soaking), percolation, counter-current extraction, pressurized liquid extraction, and soxhlet are widespread technologies described to extract bioactive compounds (Pasqueta et al., ۲۰۱۱). Consequently, more innovative and rapid techniques, such as supercritical fluid extraction, ultrasound-assisted extraction (Wang and Weller, ۲۰۰٦), microwave-assisted extraction (MAE) and pressurized solvent extraction (PSE) (Filly et al. ۲۰۱٤) have been investigated.

MAE based methods seem to present some advantages when compared to other traditional methods. The advantages of using microwave heating in comparison with conventional methods include a shorter operational time, minimal sample manipulation for extraction process, faster energy transfer, reduced thermal gradients within the matrix, good reproducibility and higher quality and quantity of the extract (Ondruschka and Asghari, ۲۰۰٦). This technique has already been demonstrated to be suitable for extracting a number of analytes from different plant materials (Zekovic et al. ۲۰۱٦) and microalgae such as Spirulina (Kalsum, ۲۰۱۹). MAE efficiency is largely affected by solvent composition or ethanol concentration, solvent volume, and microwave power and extraction time are to be considered during extraction of plant phyto-constituents (Setyaningsih et al., ۲۰۱٥). Microwave consists of electromagnetic field which oscillates in frequency range from ٠/٣ to ٣٠٠ GHz or between wavelengths of ١ cm and ١m. These

electromagnetic waves are made up of two oscillating perpendicular fields: electrical field and magnetic field. Microwave can penetrate into herbal materials and interact with the polar compounds, causing heat generation. The heating of microwave energy acted directly on molecules by ionic conduction and dipole rotation (Zhang et al. ۲۰۱۱). MAE consists of heating the solvent in contact with the sample by means of microwave energy. The process involves disruption of hydrogen bonds, as a result of microwave-induced dipole rotation of molecules, and migration of the ions, which enhance penetration of the solvent into the matrix, allowing dissolution of the components to be extracted. (Mandal et al.,۲۰۰۷).

In this study, the main objective was to optimize microwave technology conditions for the extraction of bioactive compounds from *S.platensis* extract. RSM was designed to systemic analyze the effects of extraction parameters on the TPC and TFC from *S.platensis* extract and their interactions.

Materials and Methods

Absolute ethanol were obtained from Merck Company (Germany), and Folin-Ciocalteu reagent, and ۲-۲-diphenyl-۱-pycril hydrazine (DPPH) were purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). All other chemicals were of analytical grade.

Preparation of the samples

This study utilized laboratory-scale microwave irradiation for sample preparation. Five grams of the sample were mixed with varying ratios of sample to solvent. The mixture was subsequently exposed to microwave irradiation at different time intervals (٦٠, ٩٠, and ١٢٠ sec) with a power of ١٥٠ W. To regulate temperature, the irradiation was applied intermittently; after each minute of microwave exposure, the sample was cooled in a refrigerator until the temperature dropped below ٣٠° C. For optimal MAE, the sample was immersed in the solvent for ٩٠ min without agitation. Finally, the resulting extract was separated from plant materials using filter paper (Pan et al., ٢٠٠٠).

Determination of Total Phenolic Content (TPC)

Determination of TPC was conducted according to the method described by Sun et al. (٢٠٠٧) and its modified version with slight modifications based on the method by Lu et al. (٢٠٠١). In brief, ١,٠ milliliter of ethanol extract was mixed with ٧٥,٠ ml of ١٠٪ Folin-Ciocalteu reagent. After ١٠ min, ٧٥,٠ ml of ٢٪ sodium carbonate solution was added, and the mixture was left in darkness for ٤٥ min for absorption. The absorbance was measured at ٧٦٥ nanometers wavelength. TPC was determined using a calibration curve prepared from gallic acid standards. Results were expressed as milligrams of gallic acid equivalents per gram of spirulina dry weight (Lu et al., ٢٠١١).

Determination of Total Flavonoid Content (TFC)

The TFC was determined using the aluminum chloride colorimetric method. Specifically, ٠,٥ ml of extract was mixed with ١,٠ ml of ١٠٪ aluminum chloride, ١,٠ ml of potassium acetate, and ٨,٢ ml of distilled water. After incubating the mixture for ٣٠ min at room temperature, the absorbance was measured at ٤١٥ nanometers wavelength. Quercetin served as the standard, and results were reported as milligrams of quercetin equivalents per gram of spirulina dry weight. A calibration curve was established using absorbance readings from various concentrations of quercetin (Chang et al., ٢٠٠٢).

Determination of DPPH Radical Scavenging Activity

The DPPH (١, ١-diphenyl ٢-picrylhyorazyl) free radical scavenging activity of *S. platensis* water extracts was determined according to the method described by Choi et al. (٢٠٠٠). Butylated

hydroxytoluene (BHT) was used as a reference result. The radical scavenging activity of *S. platensis* water extracts were expressed as (%) inhibition of DPPH. Reading was calculated according to the following equation of Yen and Duh (١٩٩٤):

$Ip = A_B - A_A \div A_B$ (١)

Where:

Ip: Inhibition percentage, AB: Absorbance values of the blank samples checked after ٧٠ min, AA: Absorbance values of *S. platensis* solutions checked after ٧٠ min.

Validation of the Model

The independent variables of the process included the ethanol content (or ethanol-water ratio) (%:$X_١$) and extraction time (sec:$X_٢$). In other words, the sum of the ethanol and water as solvent was considered to be ١٠٠٪ in the experiments and for the calculations by RSM. The treatments were determined in the form of ethanol content (٠–١٠٠٪v/v) and extraction time (٦٠-١٢٠ sec) by Minitab software (Table ٢). The model used in the RSM is generally a quadratic equation. For each dependent variable, a model is defined to determine the critical and interaction effects of factors on each individual variable. The multivariate model is given in equation ٢, where Y is the predicted response, $\beta_٠$ is the constant coefficient, $\beta_١$ and $\beta_٢$ are linear coefficients, $\beta_{١١}$ and $\beta_{٢٢}$ are square effects and $\beta_{١٢}$ and $\beta_{٢١}$ are interactions.

$Y = \beta_٠ + \beta_١X_١ + \beta_٢X_٢ + \beta_{١٢}X_١X_٢ + \beta_{١٢}X_{١٢} + \beta_{٢٢}X_{٢٢}$ (٢)

In this study, the central composite design was selected to optimize the process variables in ٢ levels with ١٣ components, including ٦ replicate at the central point for the estimation of the experimental error. The ranges and levels of independent variables are presented in Table ١. Experimental data were processed with a quadratic polynomial model, indicating the TFC efficiency and DPPH radical scavenging activity as a function of independent variables. The statistical significance test was based on total error with ٩٥ % confidence level ($P<٠,٠٥$).

Table ١. Experimental central composite design for the optimization of extraction of *S.platensis* extract.

Trearments	$X_١$	$X_٢$	TPC (mg/g)	TFC (mg/g)	$IC_{٥٠}$ (mg/g)
١	٥٠	٦٠	٢,٢٤٣٠٦٨٦	٠,٧٥٥٢٦٣١٥٨	١,٠٤٣٩٤٨١٢٧
٢	١٤,٦٤٥	٦٨,٧٨٧	١,٧٤٨٣٦٢٢	٠,٦٦٥٧٨٩٤٧٤	١,٤٦٨٢٩٧٦٢٦
٣	٥٠	٩٠	١,٨٦٣٥٦٧٨	٠,٧٣٠٧٠١٧٥٤	١,٣٢٠٠١٥٦١٧
٤	١٤,٦٤٥	١١١,٢١٣	١,٦٧٣٨١٧٤	٠,٦١٦٦٦٦٦٦٧	١,٤٠٧٩٦٧٧٩٦
٥	٥٠	٩٠	١,٨٣٦٤٦٠٦	٠,٤٩٣٨٥٩٦٤٩	١,٧٠٢٩٨٨٥٨٣
٦	٥٠	٩٠	١,٨٥٦٧٩١	٠,٥٧١٠٥٢٦٣٢	١,٧٤٢٧٨٣٥٠٥
٧	١٠٠	٩٠	١,٩٦٥٢١٩٨	٠,٥٥٣٥٠٨٧٧٢	١,١٣٩٨٣٠٨٠٥
٨	٥٠	١٢٠	١,٧٨٢٢٤٦٢	٠,٥٥٣٥٠٨٧٧٢	١,٦٤١٢٦٢١٣٦
٩	٨٥,٣٥٥	١١١,٢١٣	٢,٠٥٣٣١٨٢	٠,٦٦٥٧٨٩٤٧٤	١,٥٠٤٨٩٦١٤٢
١٠	٥٠	٩٠	١,٨٩٠٦٧٥	٠,٥٤٤٧٣٦٨٤٢	١,٧٢٨٥٢٧٦٠٧
١١	٠	٩٠	١,٨٥٠٠١٤٢	٠,٦٠٢٦٣١٥٧٩	١,٢٦٢١٩٥١٢٢
١٢	٨٥,٣٥٥	٦٨,٧٨٧	٢,١٢٧٨٦٣	٠,٥٤٦٤٩١٢٢٨	١,٤٦١٥٢٧٣٧٨
١٣	٥٠	٩٠	١,٩٦٥٢١٩٨	٠,٦٥٨٧٧١٩٣	١,٧٦٤٦١٣٧٧٩

$X_١$: ethanol content (% v/v); $X_٢$: time (sec)

Statistical analysis

In this study, Microsoft Office ٢٠٢٠ software was used to draw tables and charts and software Minitab version ١٩ was used to analyze the data and draw optimization charts. The levels of the independent variables are presented in actual and coded form in Table ٢. All experiments were run in triplicate. The deviation from the mean at the ٩٥٪ confidence level was employed to determine the differences in results.

Table ٢. Independent variable values of the process and their corresponding levels.

Independent variable	Symbol	-١	٠	+١

Ethanol (% v/v)	X_1	۰	۵۰	۱۰۰
Time (Sec)	X_2	۶۰	۹۰	۱۲۰

Selection of an appropriate model

According to Table ۱, the highest recorded TPC was ۲۴,۲ mg GAE/g, followed by ۷۵,۰ mg RE/g for TFC, and the lowest IC_{50} value (indicating the highest radical scavenging activity of the extract) was ۱,۰۴ mg/g. Experimental data were analyzed using a second-degree polynomial model, which describes the relationship between TPC and DPPH radical scavenging activity as functions of independent variables. Statistical significance was determined based on the overall error with a confidence level of ۹۵٪ ($p < ۰,۰۵$). The optimal range for the ethanol-water solvent ratio (۱۰۰-۰٪ v/v) and extraction time (۶۰-۱۲۰ Sec) was determined based on preliminary experiments (see Table ۱). As shown in Table ۳, the coefficient values of the model used for TPC, TFC, and IC_{50} indicate a good fit of the model to the experimental data. Notably, the model exhibited the highest accuracy in predicting total phenolic content in spirulina extracts among the variables examined (Table ۳).

Table ۳. Analysis of variance (ANOVA) response surface model models.

Source	Model	R^2	R^2-adj
TPC (mgGAE/g)	$Y_1=۱/۸۸۲۵۴+۰/۱۱۵۲۴(X_1)-۰/۱۰۰۱۰(X_2)-۰/۱۰۰۰۰(X_1X_2)-۰/۰۰۲۲۹(X_1^2)$ $+۰/۰۵۰۲۳(X_2^2)$	۹۱/۳۱	۸۵/۲۳
TFC (mg RE/g)	$Y_2=۰/۵۹۹۸۲۵-۰/۰۱۷۴۵۶(X_1)+۰/۰۲۶۸۹۴(X_2)+۰/۰۴۲۱۰۵(X_1X_2)-$ $۰/۰۰۹۰۱۳(X_1^2)-۰/۰۲۹۱۴۵(X_2^2)$	۸۰/۵۶	۷۴/۱۳
IC_{50} (mg/g)	$Y_2= ۱/۶۵۱۷۹-۰/۰۱۰۳۶(X_1)+۰/۱۰۳۴۷(X_2)+۰/۰۲۵۹۲(X_1X_2)-۰/۱۷۸۱۷(X_1^2)-$ $۰/۱۰۷۳۷(X_2^2)$	۷۵/۰۶	۷۰/۱۱

Results and Discussion

Analysis of response surface the the TPC

Table ۴ presents the experimental and predicted values of TPC, showing significant effects of various extraction conditions. The TPC in *S. platensis* ranged from ۱,۶۷ to ۲,۴۲ mg/g, with no significant difference found between the experimental and software-predicted values ($p > ۰,۰۵$).Table ۵ displays the results of variance analysis for optimizing TPC extraction from spirulina bioactive compounds. The model used demonstrated a significant fit at a ۹۵٪ confidence level ($p< ۰,۰۵$) for linear effects of ethanol (X_1) and time (X_2). However, second-degree effects such as ethanol × ethanol (X_{12}), time (X_{22}), and the interaction effect of ethanol and time (X_1X_2) were not significant ($p > ۰,۰۵$). In this study, extraction time significantly influenced the TPC of spirulina extracts using microwave-assisted methods, consistent with findings by Peng et al. (۲۰۲۳). Ya-Qin et al. (۲۰۰۹) investigated temperature and time effects on phenolic compound extraction from citrus fruits, noting increased extraction at higher temperatures and longer times, followed by a significant decline under extreme conditions. The optimal extraction conditions for maximum TPC were found between ۶۰-۶۵ sec and ethanol concentrations of ۸۰-۱۰۰٪. Decreasing ethanol levels below ۸۰٪ and extending extraction time beyond ۶۰ sec significantly reduced the TPC ($p < ۰,۰۵$) (See figures a and b).

Table ۴. TPC of *S.platensis* in different conditions.

Treatments	TPC (mg/g)	Predicted TPC (mg/g)
۱	۲/۲۴۳	۲/۱۲۵
۲	۱/۷۴۸	۱/۹۱۵
۳	۱/۸۶۴	۱/۸۸۳
۴	۱/۶۷۴	۱/۷۱۵
۵	۱/۸۳۶	۱/۸۸۳

٦	١/٨٥٧	١/٨٨٣
٧	١/٩٦٥	٢/٠٤١
٨	١/٧٨٢	١/٨٤١
٩	٢/٠٥٣	١/٩٤٦
١٠	١/٨٩١	١/٨٨٣
١١	١/٨٥٠	١/٧١٥
١٢	٢/١٢٨	٢/١٤٦
١٣	١/٩٦٥	١/٨٨٣

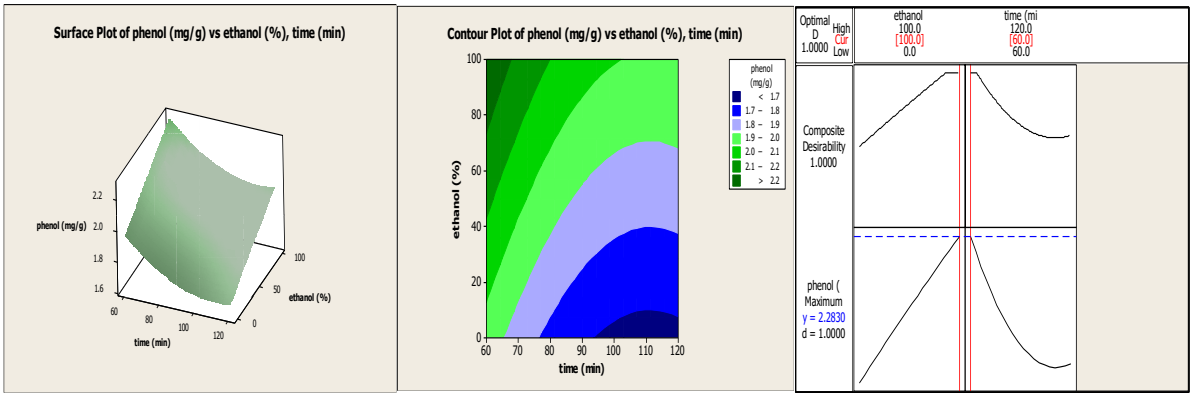
These results indicate that while longer extraction times initially increase phenolic compound yield, exceeding optimal time thresholds results in a significant decline in extracted phenolics. This decline may be attributed to polymerization reactions among phenolic compounds at higher temperatures, as reported in previous studies (Pinelo et al., ٢٠٠٥). Figure c illustrates the optimal conditions for extracting TPC from spirulina using microwave-assisted methods, achieving a peak of ٢٨,٢ mg GAE/g at an ethanol ratio of ١٠٠:٠ and an extraction time of ٦٠ sec. Thus, employing a higher ethanol ratio (١٠٠٪) resulted in the most efficient extraction of phenolic compounds, likely due to enhanced solvent penetration into plant tissues and subsequent disruption of cell wall polysaccharides, facilitating compound release. Additionally, the solvent's polarity plays a crucial role in phenolic compound extraction efficiency, with ethanol demonstrating superior affinity compared to other solvents.

Table ٥. Analysis of variance (ANOVA) response surface model of TPC.

Source	DF	F value	P value (Prob > F)
Regression	٥	٣/٠٨	٠/٠٨٨
Linear	٢	٧/٠١	٠/٠٢١*
Ethanol (%,v/v)($X_١$)	١	٧/٩٩	٠/٠٢٦*
Time (sec)($X_٢$)	١	٦/٠٣	٠/٠٤٤*
Square	٢	٠/٦٨	٠/٥٣٧
Ethanol(%,v/v)×ethanol(%,v/v) ($X_١^٢$)	١	٠/٠٠	٠/٩٦٠
Time (sec)×time (sec)($X_٢^٢$)	١	١/٣٢	٠/٢٨٨
Interaction	١	٠/٠٠	٠/١
Ethanol(%,v/v)×time (sec)($X_١X_٢$)	١	٠/٠٠	٠/١

*Significant effect (P<٠/٠٥).

Moreover, ethanol, as the solvent used in this study, has been noted by researchers for its superior ability to extract phenolic compounds compared to many other solvents. The polarity of solvents significantly influences phenolic compound extraction efficiency, with these compounds generally being bulky and of low polarity. Water-based extractions typically yield lower quantities of phenolic compounds compared to solvents like methanol and ethanol, owing to water's high dielectric constant and low loss factor (Proestos et al., ٢٠٠٨). Therefore, ethanol concentrations of ١٠٠٪ yielded the highest TPC in present study, owing to its superior ability to extract these compounds efficiently.



(a) (b) (c)

Figure ١. The contour (a) and surface (b) plot and optimal conditions (c) for TPC (mg/g) vs time (min), ethanol (%).

Analysis of response surface the the TFC

According to Table ٦, the TFC in spirulina extracts ranged from ٠,٥٥ to ٠,٧٥ mg/g, with no significant difference found between experimental and software-predicted values ($p > ٠,٠٥$). Analysis of variance in Table ٧ indicated that the second-degree polynomial model was not statistically significant ($p > ٠,٠٥$). The coefficient of determination (R-Sq = ٨٠,٥٦٪) and adjusted coefficient (R-Sq (adj) = ٧٤,١٣٪) for this model showed a satisfactory fit to the experimental data (Table ٣). Figure ٢ illustrates the three-dimensional (a), contour plot (b), and optimal surface plots (c) of flavonoid content (mg RE/g) in relation to extraction time (sec) and ethanol concentration (%, v/v), demonstrating significant effects ($p < ٠,٠٥$). Increasing the extraction time up to ٦٠ sec and reducing ethanol concentration to ٠٪ (١٠٠٪ water) significantly increased TFC ($p < ٠,٠٥$). This suggests that a considerable portion of the compounds in spirulina extracts are highly polar or associated with sugars or other polar groups. Extraction times shorter or longer than ٦٠ sec resulted in a significant decrease in TFC. Consistent with these findings, Soroush et al. (٢٠٢١) observed that extending the extraction time of spirulina extracts enhances flavonoid extraction and overall extraction efficiency.

Table ٦. TFC of *S.platensis* in different conditions.

Treatments	TFC (mg/g)	Predicted TFC (mg/g)
١	٠/٧٥٥	٠/٦٩٦
٢	٠/٦٦٦	٠/٧٠٦
٣	٠/٧٣١	٠/٦٠٠
٤	٠/٦١٧	٠/٥٦٨
٥	٠/٤٩٤	٠/٦٠٠
٦	٠/٥٧١	٠/٦٠٠
٧	٠/٥٥٤	٠/٥٥٧
٨	٠/٥٥٤	٠/٦٢٠
٩	٠/٦٦٦	٠/٦١٨
١٠	٠/٥٤٥	٠/٦٠٠
١١	٠/٦٠٣	٠/٦٠٦
١٢	٠/٥٤٦	٠/٥٨٧
١٣	٠/٦٥٩	٠/٦٠٠

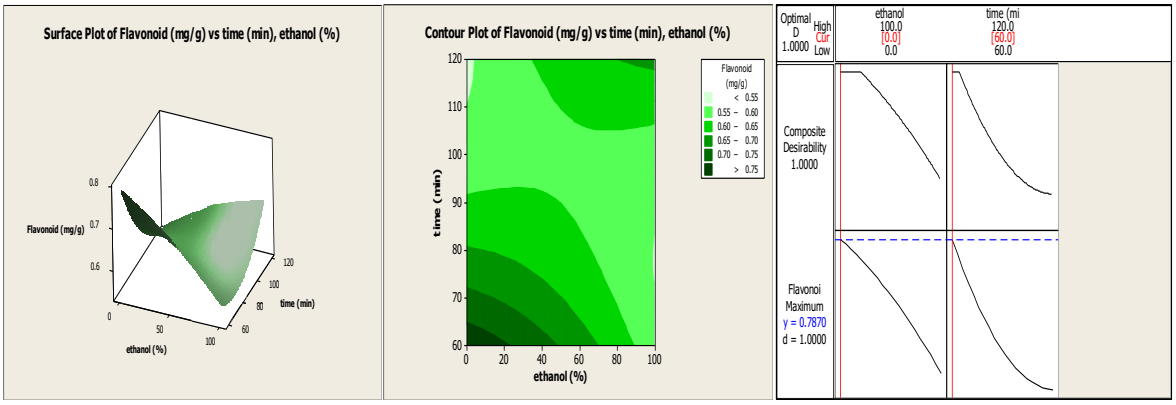
In this study, optimal conditions for achieving the highest TFC in spirulina extracts (٠,٧٥ mg RE/g) were determined as a ٦٠-sec extraction time with ٠٪ ethanol (١٠٠٪ water) (Figure ٢).

Table ٧. Analysis of variance (ANOVA) response surface model of TFC.

Source	DF	F value	P value (Prob > F)

Regression	٥	٠/٦١	٠/٦٩٩
Linear	٢	٠/٥٦	٠/٥٩٦
Ethanol (%,v/v)(X_1)	١	٠/٣٣	٠/٥٨٣
Time (sec)(X_2)	١	٠/٧٨	٠/٤٠٥
Square	٢	٠/٤٨	٠/٦٣٨
Ethanol(%,v/v)×ethanol(%,v/v) (X_1^2)	١	٠/٠٨	٠/٧٩٠
Time (sec)×time (sec)(X_2^2)	١	١/٨٠	٠/٤٠٠
Interaction	١	٠/٩٦	٠/٣٥٩
Ethanol(%,v/v)×time (sec)(X_1X_2)	١	٠/٩٦	٠/٣٥٩

*Significant effect (P<٠/٠٥).



(a) (b) (c)

Figure ٢. The contour (a) and surface (b) plot and optimal conditions (c) for TFC(mg RE/g) vs time(sec), ethanol (%).

The analysis of response surface of IC٥٠ value (DPPH method)

According to Table ٨, the radical scavenging activity of spirulina extracts ranged from ١,٤٤ to ٧,٦١ mg/g and showed no significant difference between experimental and software-predicted values (p > ٠,٠٥). Analysis of variance in Table ٩ for the second-degree polynomial model indicated that the predicted model was not statistically significant (p > ٠,٠٥). The coefficient of determination (R-Sq = ٧٥,٠٦٪) and adjusted coefficient (R-Sq (adj) = ٧٠,١١٪) suggested a poor fit of the model to the experimental data (Table ٣). As shown in Figure ٢, extraction time and solvent ratio (ethanol to water) significantly influenced radical scavenging activity (p < ٠,٠٥). Increasing the extraction time beyond ٦٠ sec and reducing ethanol concentration to less than ٩٥٪ resulted in an increase in IC٥٠ values, indicating a decrease in maximum radical scavenging activity. Specifically, ethanol concentrations of ٩٠ -١٠٠٪ (٠-١٠٪ water) and a ٦٠-sec extraction time yielded the lowest IC٥٠ values (highest radical scavenging activity). Tong et al. (٢٠١٢) reported that the increase in the extraction time increases the reactive site to the effective extraction process, which enhances the antioxidant activity of spirulina extract.

Optimal conditions for achieving the highest radical scavenging activity in spirulina extracts (٧,٦١ mg/g) were determined as a ٦٠ sec extraction time and ١٠٪ ethanol (٩٠٪ water) (Figure ٣c). Regarding the type and ratio of solvent, these findings contradict Mandal et al. (٢٠٠٧), who suggested that higher ratios in MAE may result in inadequate solvent penetration due to insufficient and improper mixing. Generally, the difference in antioxidant activity among various extracts stems from the different polarities of the solvents used, affecting the extraction efficiency of bioactive compounds (Marinova and Yanishlieva, ١٩٩٧). In this study, a direct relationship was observed between TPC and radical scavenging activity (IC٥٠). This relationship is consistent with the findings of Fang et al. (٢٠٠٩) in their study on the antioxidant properties of Bayberry extracts.

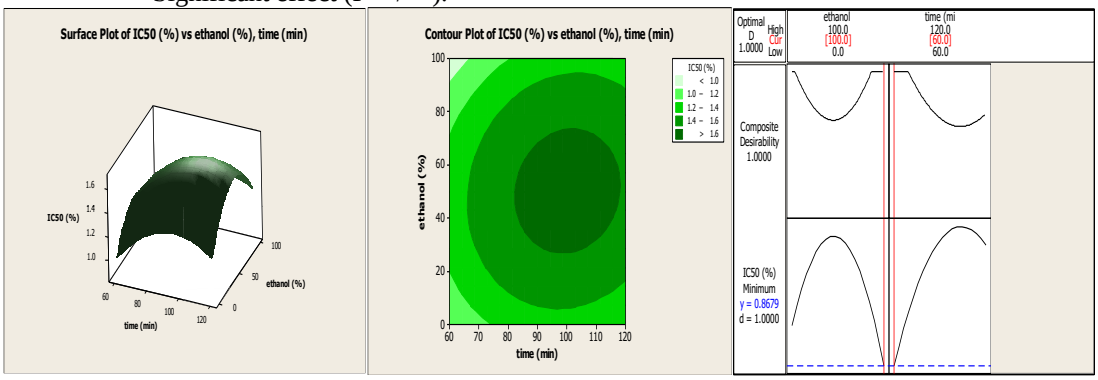
Table ٨. IC٥٠ values of *S.platensis* in different conditions.

Treatments	IC_{50}. (mg/g)	Predicted IC_{50}. (mg/g)
١	١/٠٤٤	١/٢٩١
٢	١/٤٦٨	١/٢٩٩
٣	١/٣٢٠	١/٦٥٢
٤	١/٤٠٨	١/٤٥٤
٥	١/٧٠٣	١/٦٥٢
٦	١/٧٤٣	١/٦٥٢
٧	١/١٤٠	١/٢٨١
٨	١/٦٤١	١/٥٨٣
٩	١/٥٠٥	١/٤٨٥
١٠	١/٧٢٩	١/٦٥٢
١١	١/٢٦٢	١/٣١٠
١٢	١/٤٦٢	١/٢٢٦
١٣	١/٧٦٥	١/٦٥٢

Table ٩. Analysis of variance (ANOVA) response surface model of TFC.

Source	DF	F value	P value (Prob > F)
Regression	٥	٠/٦١	٠/٦٩٩
Linear	٢	٠/٥٦	٠/٥٩٦
Ethanol (%,v/v)(X_1)	١	٠/٣٣	٠/٥٨٣
Time (sec)(X_2)	١	٠/٧٨	٠/٤٠٥
Square	٢	٠/٤٨	٠/٦٣٨
Ethanol(%,v/v)×ethanol(%,v/v)(X_1^2)	١	٠/٠٨	٠/٧٩٠
Time (sec)×time (sec)(X_2^2)	١	١/٨٠	٠/٤٠٠
Interaction	١	٠/٩٦	٠/٣٥٩
Ethanol(%,v/v)×time (sec)(X_1X_2)	١	٠/٩٦	٠/٣٥٩

*Significant effect (P<٠/٠٥).



(a) (b) (c)

Figure٣.The contour(a) and surface(b) plot and optimal conditions(c) for IC_{50} values (mg/g)vs time (sec), ethanol (%).

In contrast, in some studies, DPPH values are not significantly correlated with TPC. Meneses et al. (٢٠١٣) reported a very low and weak correlation between these two parameters in brewers' spent grain. It should be pointed out that the Folin-Ciocalteau method can measure compounds other than phenols and final absorbance could also be the result of other reduction reactions. On the other hand, the antioxidant activity of a plant extract cannot only be related to its TPC. Other specific phenolic compounds such as flavonoids, tannins, proanthocyanidins, and amino phenolic compounds can play a more important role in antioxidant activity (Andres et al., ٢٠٢٠). These findings highlight the importance of using a sufficient volume of solvent to ensure complete immersion of plant tissues during irradiation (Mandal et al., ٢٠٠٧). Depending on the extraction method and solvent used, the extraction efficiency of bioactive compounds and the antioxidant

activity of the extracts may vary. The type of solvent significantly affects the extraction of active plant compounds, as molecules with antioxidant properties in polar environments often transform into more stable acidic ions compared to hydroxyl ions, thereby enhancing their antioxidant stability (Hosseinzadeh et al., ۲۰۰۹).

Conclusions

According to results, analysis of variance (ANOVA) shows that second-order polynomial model provided adequate mathematical description of the MAE of polyphenols with high antioxidant activity. The extraction time and solvent ratio had high significant effects on the response values, followed by the significant interaction effects between the extraction time and ratio of water to ethanol. A high correlation of the quadratic polynomial mathematical model was gained and could be great employed to optimize antioxidants extraction from *S.platensis* by microwave. The optimal extraction conditions for the natural bioactive compounds were determined as follows:

The ethanol content ۱۰۰٪ (v/v) and extraction time ٦۰ sec for the TPC (۲٫۲۸ mg GAE/g) and $IC_{۵۰}$ value (۱٫۰٤ mg/g) and water content ۱۰۰٪ (v/v) and extraction time ٦۰ sec for the TFC (۰٫۷۵ mgRE/g). No significant differences were observed between the predicted and experimental values (P>۰٫۰۵) which indicated that the experimental results confirmed adequate fitness of the predicted model. These results show that using relatively high ethanol concentration and during short extraction time, a potent natural extracts can be obtained.

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Symbols:

RSM: Response surface methodology
TPC: Total phenolic content
TFC: Total flavonoid content
GAE: Gallic acid equilibirum
RE: Rutin equilibrium
MAE: Microwave-assisted extraction
PSE: Pressurized solvent extraction
GHz: Giga Hertz
DPPH: ۲-۲-diphenyl-۱-pycril hydrazine
BHT: Butylated hydroxytoluene
Sec: Second
Min: Minute

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