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Original Article

Development and Optimization of Novel Microwave-Assisted Extraction for Rich-Flavonoids and Polyphenols from *Dioscorea bulbifera* Leaves by Response Surface Methodology

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ABSTRACT

Objectives: Microwave-Assisted Extraction (MAE) has emerged as a promising technique for the extraction of bioactive compounds from plant materials due to its efficiency and reduced processing time. In the present study, variables of MAE were optimized for maximizing the extraction of polyphenolic and flavonoid constituents from *Dioscorea bulbifera* leaves by Box-Behnken Design (BBD) of response surface methodology. **Materials and Methods:** Box-Behnken Design (BBD) was utilized to find the optimal microwave extraction condition for obtaining extracts enriched with polyphenolic compounds. The independent variables for BBD are irradiation power (400-800W), time (1-5 min) and concentration of ethanol (20-60%). Total phenolic content and total flavonoid content were estimated by the Folin ciocalteu method and aluminium chloride colorimetric assay respectively. Using qualitative chemical testing and FTIR analysis, the phytoconstituents and functional properties of the extracts were determined. **Results:** The ANOVA results obtained from the design of expert software exhibited that Total Phenolic Content (TPC) and total flavonoid content were significantly (p<0.0001) affected by microwave power of irradiation, time and concentration of ethanol, indicating good agreement between the experiment and predicted values. The ideal parameter of MAE for maximum yield of TPC (3.49 mg GAE/g) and TFC (0.61 mg QE/g) were 400W of microwave power, 5 min of irradiation time and 48.467% ethanol concentration. **Conclusion:** The optimized extracts can be used as a source of antioxidants with potential applications in functional foods, dietary supplements and pharmaceutical formulations aimed at combating oxidative stress-related diseases.

Keywords: Optimization, Polyphenols, Microwave, Box-Benkhen design.

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INTRODUCTION

Dioscorea bulbifera (family Dioscoreacea) popularly known as air potato, or bitter yam is a traditional medicinal plant native to America, Northern Australia, Asia and tropical Africa.[1] Traditionally, *Dioscorea bulbifera* tubers have been used widely to treat several diseases, such as goiter, hemoptysis, skin infections, pharyngitis, orchitis, cancer, dysentery and tuberculosis.[2-4] The paste of the leaves of *Dioscorea bulbifera* was used in the treatment of skin diseases and to remove dandruff.[5] Dioscorea contain important phytochemicals such as diosbulbins, flavonoids, carotenoids, steroidal saponins, essential amino acids and phenolic compounds. Dioscorea is rich in phenolic and flavonoid compounds which act as a source of natural antioxidants which has great potential due to their free radical scavenging activity in the treatment of various diseases. Phenolic compounds are relatively thermolabile and prone to oxidizing at high temperatures over an extended period. The extraction of phenolic compounds and flavonoids is usually performed by conventional maceration method using different types of solvents.[6] However, this extraction technique consumes high amounts of solvent, requires long extraction times and imposes a high risk of degrading the heat-labile constituents in the sample. To overcome these problems, one of the suitable extraction methods that can be used is Microwave Microwave-Assisted Extraction (MAE) method.

Microwave-Assisted Extraction (MAE) involves the use of microwaves to produce heat by interacting with polar compounds and a few organic components in the plant matrix, after the ionic

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conduction and dipole rotation mechanisms. MAE is widely used for quick extraction of analytes, especially for thermally unstable compounds, due to its faster heating of sample solvent mixture due to the use of microwave.[7]

The efficiency of MAE depends on several factors, including solvent composition, microwave power, extraction temperature, solvent-to-solid ratio, sample particle size and preleaching time.[8] For studying an extraction process with many variables, there is a need for a suitable experimental design that can generate optimum conditions from the least number of experiments. Response Surface Methodology (RSM) is a useful technique for studying the effects of individual factors as well as their interactions.[9] One of the designs of an experiment for RSM is Box-Behnken Design (BBD) which needs fewer design points to study the processes with a minimum of three levels and helps to study processes with extreme ranges which can result in unsatisfactory results.

Phenolic compounds and flavonoids are sensitive to heat and may undergo degradation or structural changes during prolonged exposure to high temperatures.[10-12] MAE operates under milder conditions, minimizing heat-induced degradation and preserving the bioactivity of extracted compounds. This ensures that the obtained phenolic compounds and flavonoids maintain their potential health benefits and therapeutic properties. The bioactive compounds extracted from *Dioscorea bulbifera* through MAE hold potential for various industrial applications, including pharmaceuticals, nutraceuticals and functional foods. By optimizing the extraction process using MAE, researchers can obtain higher yields of phenolic compounds and flavonoids, making them more accessible for commercial production and formulation into value-added products.

Hence considering the above facts, the current study is aimed at analyzing the effect of different parameters of MAE such as microwave power, solvent concentration and time of irradiation on the yield of total phenolic and total flavonoid content of *Dioscorea bulbifera* and to optimize these parameters using Box-Behnken design.

MATERIALS AND METHODS

Materials

Gallic acid, Quercetin, Folin ciocalteu reagent were procured from Sigma Aldrich (Germany). The solvents and chemicals used were analytical grade.

Collection and preliminary processing of plant material

Fresh *Dioscorea bulbifera* leaves were obtained from the grounds of Pernem, Goa. The plant material was authenticated by Dr. Smitha Hegde, Nitte University Centre for Science Education and Research and Voucher specimen (21BP073E) deposited in the college. Leaves were washed with water to remove soil particles. Leaves were pulverized to powder form after shade drying at room temperature.

Microwave-Assisted Extraction (MAE) of plant material

Microwave-Assisted Extraction (MAE) was carried out using a microwave extractor (CATA-R, catalyst system, Pune). 5 g of *D. bulbifera* leaf powder was extracted with different concentrations of aqueous ethanol (100 mL) as a solvent at different irradiation power and time. After the extraction, extract solutions were filtered through Whatman filter paper, concentrated to dryness and the percentage yield was calculated.

Preliminary Phytochemical Analysis

Various standard chemical tests were performed to determine various phytoconstituents in the prepared extracts.[13]

Optimization of MAE

To optimize the MAE conditions, DOE was performed using Design Expert® software by response surface methodology in which Box-Behnken design was used. For the optimization of MAE, three factors and three levels of BBD were applied by choosing microwave power., time and concentration of ethanol as independent variables and Total Phenolic Content (TPC) content, Total Flavonoid Content (TFC) as dependent variables as shown in Table 1.

Estimation of total phenolic content

The Folin-Ciocalteau method was used for the estimation of total phenolic content in the prepared extracts.[14] The folin-Ciocalteau method involves the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids, followed by measurement of the intensity of color. The appropriate dilution of extracts is oxidized with Folin-Ciocalteau reagent and then treated with sodium carbonate. and volume is made up with distilled water. After 30 min of dark storage at room temperature, the intensity of the blue color was measured at 765 nm using a Shimadzu 1700 UV-visible spectrophotometer and gallic acid was used as standard. The phenolic content was calculated and shown as gallic acid equivalent per gram of the extract using the linear equation derived from the standard plot.

Preparation of standard gallic acid solutions for calibration curve: 100 mg of gallic acid monohydrate was dissolved in 100 mL phosphate buffer (pH 6.8) to give a concentration of 1000 µg/mL. From this primary stock solution 5 mL is taken and made up to 100 mL with distilled water to give a concentration of 50 µg/mL. From the stock solution, aliquots of 2, 4, 6, 8, 10 and 12 mL were collected and placed in 6 different 25 mL volumetric flasks. Each one was filled with 1.25 mL of the Folin-Ciocalteau reagent and 2.5 mL of 20% sodium carbonate and both were allowed to stand for 30 min and volume was made up to 25 mL

with distilled water to get a concentration in the range of 4-24 µg/mL. The absorbance of these solutions was detected at 765 nm against distilled water as blank. Using the plot of absorbance V/s concentration, a standard calibration curve was prepared, which was linear over this concentration range.

Preparation of sample solution

In 100 mL of phosphate buffer (pH 6.8), 100 mg of ethanolic extract was dissolved. From the solution mentioned above, 10 mL was extracted and 100 mL of phosphate buffer was added to dilute it. 4 mL of this were transferred to a 25 mL volumetric flask and 2.5 mL of sodium carbonate and 1.25 mL of FC reagent were added. Once distilled water had been added to make up the volume, color development was carried out for the above solutions as that for the standard and the absorbance of the sample solution was measured at 765 nm. By extrapolating the calibration graph, the total phenolic content of the test solution was estimated.

Estimation of total flavonoid content

The total flavonoid content of the prepared extract was measured by the aluminum chloride colorimetric assay.[15] Appropriate dilutions of samples, i.e., standard quercetin solution (20, 40, 60, 80 and 100 µg/mL) and 1 mL of the extract individually, were treated with 5% $NaNO_2$. After 5 min 10% AlCl3 was added, followed by the addition of 1M NaOH. Then absorbance of orange yellowish the absorbance of the orange-yellowish solution was measured against a blank at 510 nm UV-visible spectrophotometer (Shimadzu 1700). The concentration of total flavonoid was calculated based on the equation from the standard curve and expressed in terms of milligram Quercetin Equivalent per gram of the extract (mg QE/g).

Fourier transfer Infrared spectroscopy-FTIR

One of the most effective methods for determining the functional groups that are present in compounds is Fourier Transfer infrared spectroscopy. Using a Bruker FTIR spectrophotometer, the optimized ethanolic extract's FTIR spectrum was obtained.

RESULTS

Microwave-assisted extraction

The extracts obtained by MAE were green coloured dried powder and the percentage yield of the extracts obtained is shown in Table 2. The maximum yield of the extracts was obtained at 400W power with 1 min irradiation using 40% ethanol concentration.

Preliminary Phytochemical analysis

Preliminary phytochemical analysis of the extracts showed the presence of steroids, saponins, flavonoids, alkaloids, phenolic compounds, tannins and terpenoids while carbohydrates and proteins were absent.

Estimation of total phenolic content

Using gallic acid as a standard, the Folin-Ciocalteu technique was used to determine the total phenolic content in the prepared extracts. To prepare a standard plot, the absorbance of various concentrations of gallic acid solutions was measured at 765 nm. The results are shown in Figure 1 and amount of phenolic content were calculated from the equation y=0.1079x+0.0686 (R^2=0.9918)

Estimation of total flavonoid content

The total flavonoid content of prepared extracts was determined by aluminium chloride colorimetric assay using quercetin as standard. To prepare a calibration curve, the absorbance of various quercetin concentrations was measured at 510 nm. Total flavonoid content was calculated from the equation y =1.1063x-0.0325 (R^2=0.9965) obtained from standard plot as shown in Figure 2.

Optimization of MAE

Extract from *Dioscorea bulbifera* leaves were prepared by microwave-assisted extraction method. MAE variables were optimized using Box Benkhen of response surface methodology for optimal recoveries TPC and TFC. The effect of independent variables such as irradiation power (400-800W), irradiation time (1-5 min) and concentration of ethanol (20-60%) on dependable variables, i.e., total phenolic content and total flavonoid content, is given in Table 2.

Table 1: Levels and factors applied in Box-Behnken design.

Code	Independent variables	Level		
		-1	0	+1
A	Irradiation Power (W)	400	600	800
B	Irradiation Time (min)	1	3	5
C	Ethanol concentration (%)	20	40	60
	Dependent Variables	Goal		
	Total Phenolic Content (mg/GAE)	Maximize		
	Total Flavonoid Content (mg/QE)	Maximize		

Table 2: **BBD and experiment results for optimization of microwave assisted extraction from *Dioscorea bulbifera* leaves.**

Run	Factor 1 A: Power (Watt)	Factor 2 B: Time (min)	Factor 3 C: Ethanol Concentration (% V/V)	% Yield of the extract	Response 1 TPC mg GAE/g	Response 2 TFC Mg QE/g
						mg QE/g
1	600	3	40	8.8	1.08	0.23
2	400	3	20	6.4	1.09	0.38
3	400	5	40	11.6	3.49	0.6
4	800	1	40	7.2	2.53	0.4
5	800	5	40	11.6	2.08	0.4
6	400	3	60	4.2	1.42	0.36
7	600	3	40	8.8	1.01	0.21
8	600	5	60	6.6	2.01	0.45
9	600	3	40	8.8	1.18	0.25
10	600	5	20	5.6	1.15	0.41
11	800	3	20	6.4	0.71	0.34
12	400	1	40	15.6	1.81	0.26
13	600	3	40	8.8	1.01	0.21
14	600	1	60	8.6	1.56	0.3
15	600	3	40	8.8	1.11	0.22
16	800	3	60	6.8	1.45	0.33
17	600	1	20	8.4	1.09	0.34

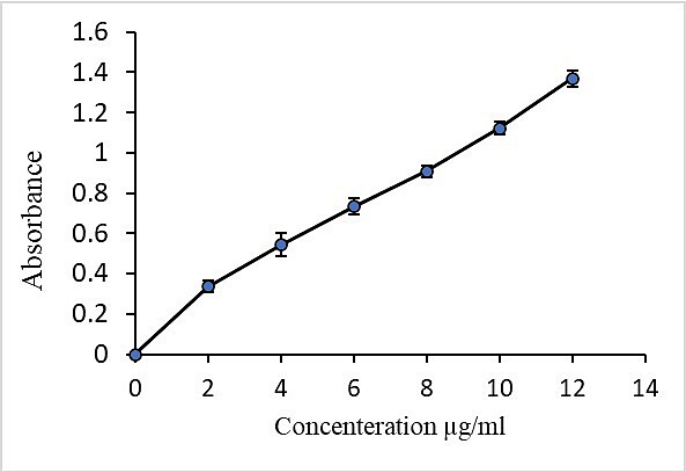

Figure 1: Calibration curve of gallic acid in phosphate buffer of pH 6.8 at 765 nm.

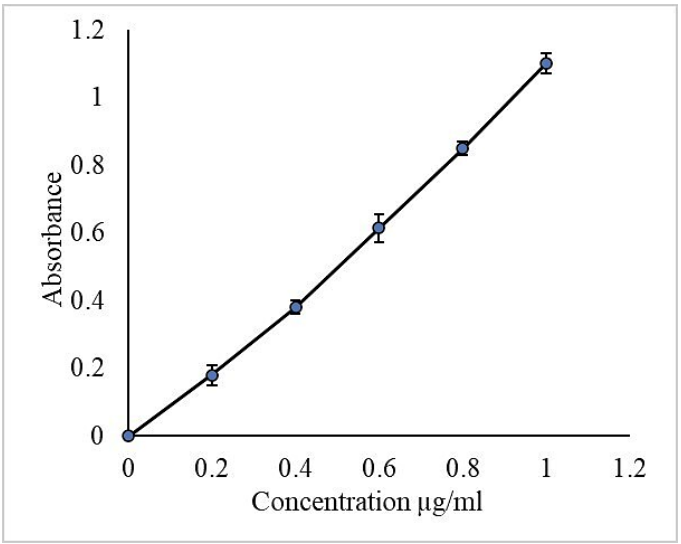

Figure 2: Calibration curve of Quercetin at 510 nm.

Table 3: **Comparison of experimental and theoretical values of responses for the validation of model established for optimization of MAE.**

Independent variables			Responses	
Power (W)	Time (min)	Ethanol (%)	TPC (mg GAE/g)	TFC (mg QE/g)
400.000	5.000	48.467	Predicted mean	
			3.399	0.600
			Observed Mean	
			3.49	0.61
% Error			2.60	1.63

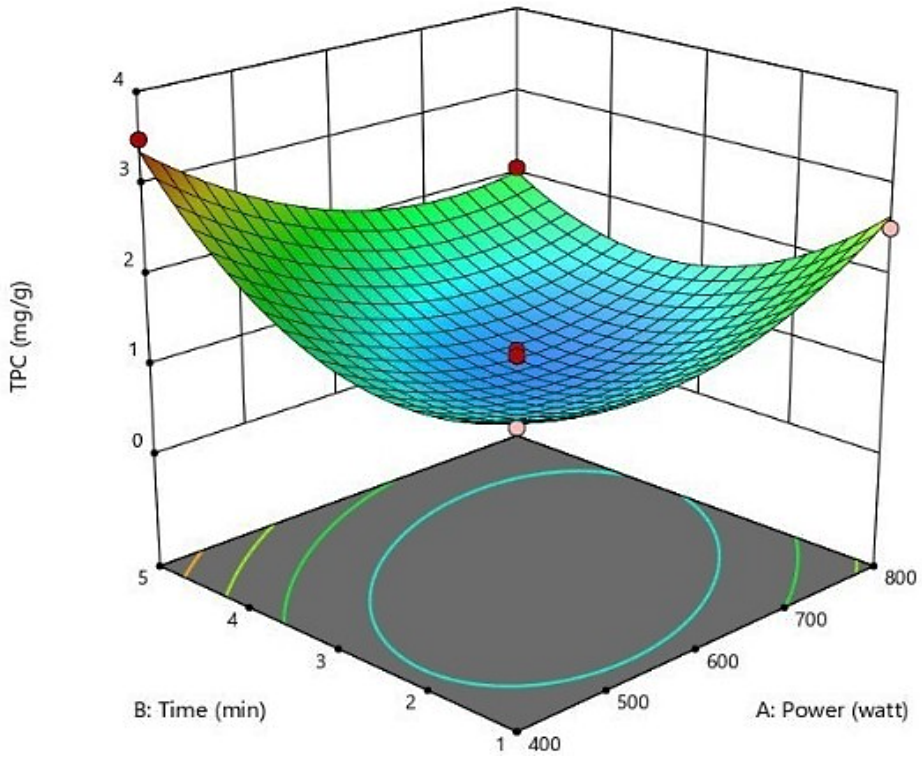

Figure 3: Response surface curve depicting the effect of microwave power, irradiation time and ethanol concentration on the yield of Total Phenolic Content (TPC).

DISCUSSION

Effect of MAE variables on total phenolic content

The extracts were found to contain total phenolic content ranging from 0.71-3.49 mg GAE/g. The effect of variables on the yield of phenolic content can be studied by applying regression analysis. Model F- value was found to be 54.59 and the quadratic model was found to be significant. The predicted R^2 is 0.8319 which agrees with adjusted R^2 of 0.9679 with the difference between them is less than 0.2. The quadratic equation generated from the analysis is given as follows:

Total Phenolic content= +1.080.10.1300A*+0.2175B*+0.3000C*0.5325AB*+0.1025AC+0.0975BC+0.573A^{2*}+0.8423B^{2*}-0.4677C^{2*}

Where A, B and C represent irradiation power, time and ethanol concentration respectively and significant variables are indicated by an asterisk sign (*). A, B, C, AB, A^2, B^2, C^2 were significant model terms (p-value<0.05). The microwave power (A), time (B) and concentration of ethanol (C) has prominent effect on the yield of total phenolic content from *Dioscorea bulbifera* leaves (p<0.05). The quadratic terms of independent variables were also significant (p<0.05). The interaction effect of microwave power and time (AB) was significant for the yield of phenolic content.

The response surface plots of the effect of microwave power, irradiation time and concentration of ethanol on the yield of total phenolic content are shown in Figure 3. The results show that low TPC yield was observed at a medium concentration of power, time and ethanol concentration while maximum yield was at a low power of 400W with a higher irradiation time of 5 min using

40% ethanol concentration. Decrease in the yield above 400W may be due to the thermal degradation of phenolic constituents present in the plant sample at higher power of irradiation of microwaves.

Heating in MAE occurs due to dipole rotation and ionic conduction which cause power dissipated into the solvent used for extraction and plant sample.[16] Absorption of microwave energy occurs in plant cell wall and results in rise in internal heating.[17] Therefore, heat produced by microwave energy may be too high to disintegrate phenolic compounds at higher power levels. Microwave and irradiation time influence each other to a greater extent on the yield TPC. Combination of low power with longer exposure is a better approach which results in a higher yield of phytoconstituents and there is always a risk of thermal degradation at high power with prolonged exposure.

Ethanol is a low polar solvent and its polarity can be increased by mixing with water. By using a mixture of binary solvents (such as ethanol and water) maximum amount of phenolic compounds can be extracted from plant samples due to the reason that their solubility relies on the polarity of solvents.[18] The amount of TPC obtained increased with the percentage of concentration of ethanol until 40% v/v and decreased beyond 40%v/v. The reason being that affinity of phenolic compounds depends on its solubility in aqueous ethanol. As the polarity of the ethanol increases with the addition of water which influences the solubility of phenolic compounds also incorporation of water increases the mass transfer between the solid and liquid by improving the penetrability of plant matrix and disrupting the bond between solute and plant matrix.[17] Therefore, the appropriate proportion of ethanol concentration should be selected. Usually, an increase in extraction time results in increase extraction of analytes, although there is a risk of thermal degradation.

Effect of MAE variables on Total Flavonoid Content (TFC)

The flavonoid content of the extract ranged from 0.21-0.60 mg QE/g of the extract. The effect of variables on the yield of flavonoid content can be studied by applying regression analysis. The quadratic model was significant with the model F- value of 42.60. The predicted R^2 value and adjusted R^2 values were 0.8083 and 0.9590 respectively. The quadratic equation generated from the analysis is given as follows:

Total Flavonoid content=+0.2240-0.0162A+0.0700B*-0.0038C -0.0850AB*+0.0025AC+0.0200BC+0.0843A^{2*}+0.1067B^{2*}+0.0443C^{2*}

where A, B and C represent microwave power, time and ethanol concentration respectively and significant variables are indicated by an asterisk sign (*). B, AB, A^2, B^2 and C^2 were found to be

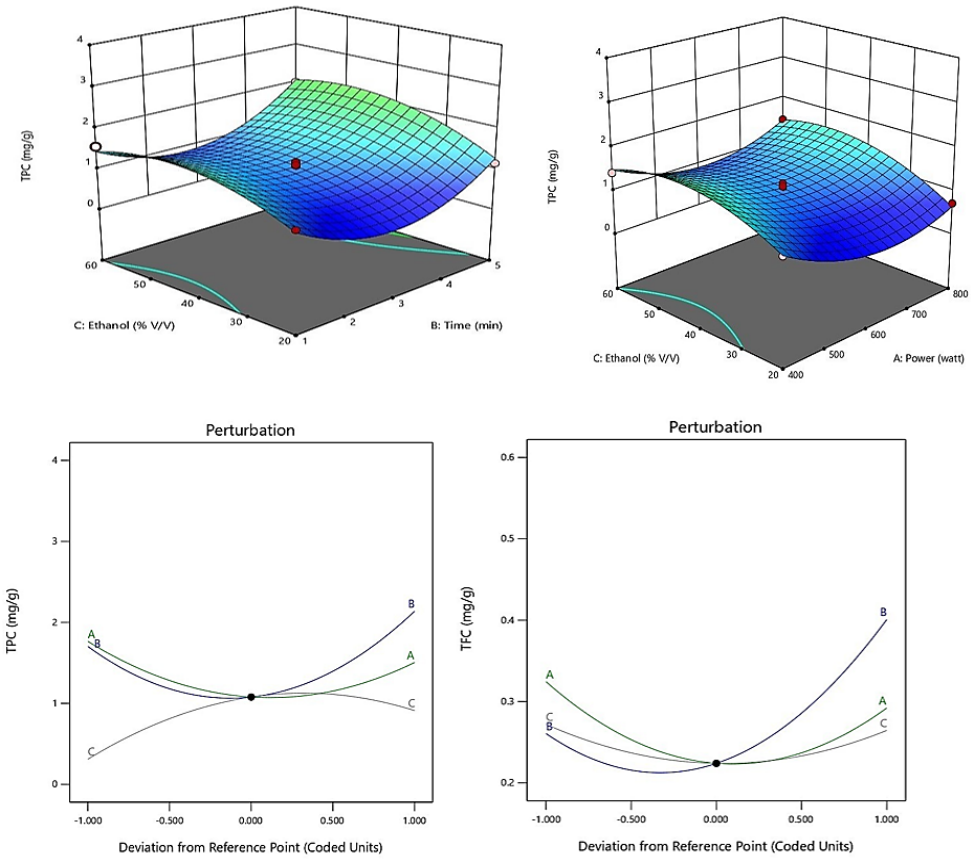

Figure 4: Perturbation graph depicting the effect of microwave power, irradiation time and concentration of ethanol on the yield of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC).

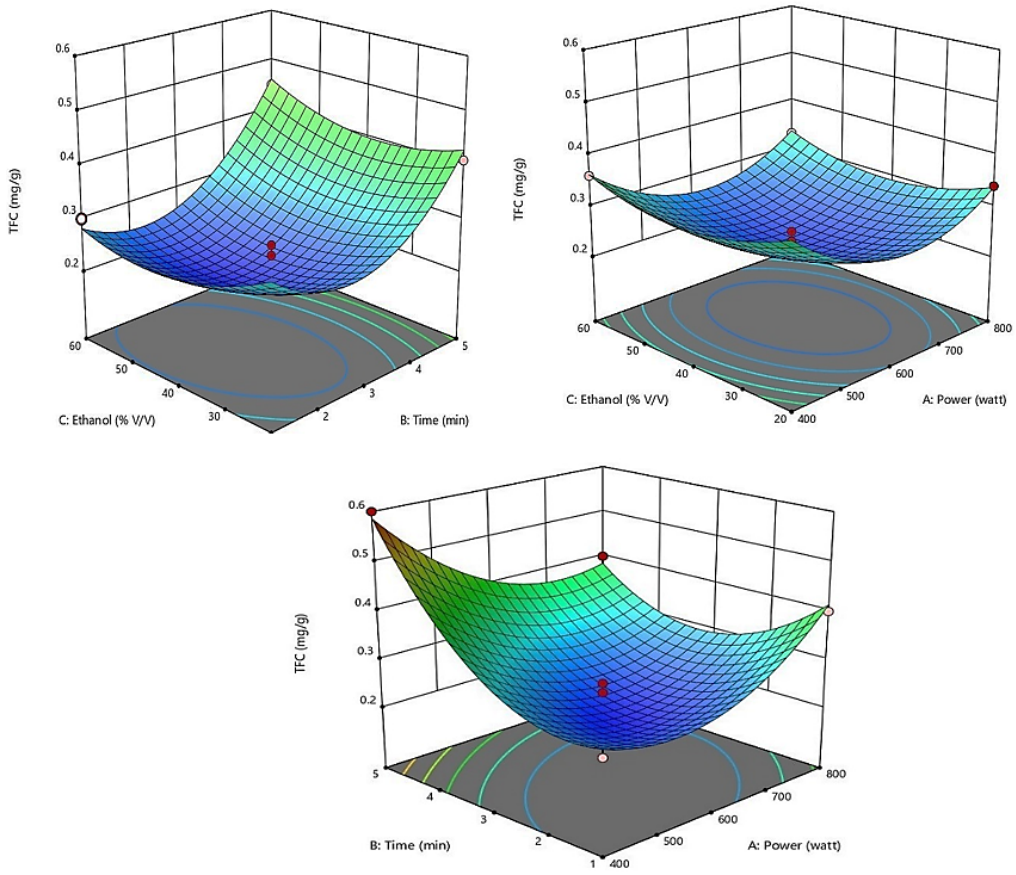

Figure 5: Response surface curve depicting the effect of microwave power, irradiation time and concentration of ethanol on the yield of Total Flavonoid Content (TFC).

significant model terms (p-value<0.05). Irradiation time (B), all the quadratic terms of the factors and the interaction effect of AB were found to have a significant effect on the recovery of flavonoids. A negative effect was observed on the yields of flavonoids with an increase in microwave power and ethanol concentration, while rise in extraction time produced a positive effect on the recovery of flavonoids which is reflected in perturbation and response surface plot shown in Figures 4 and 5 respectively.

Polarity of flavonoids were found to vary, hence solvent used for their extraction can be polar or less polar. Extraction of flavonoid aglycone is done using low polar solvent while more polar solvents are used for flavonoid glycosides and anthocyanin. Different concentrations of ethanol in aqueous solutions are commonly used as solvents for microwave-assisted extraction of both polar aglycones and flavonoid glycosides and variation in concentration of solvents has been found to affect the flavonoid extraction.[19] Maximum yield of flavonoids was observed at low power of 400w, irradiation time of 5 min and 40% ethanol concentration. Since the rate of heating with polar solvent with high dielectric properties is high, application of microwave for long periods at high power may result in degradation of phytoconstituents due to excessive heating of mixture of solute and solvent.[20] The MAE parameters were optimized based on the responses such as TPC (maximum) and TFC (maximum). The software generated several solutions in which the one with the highest desirability (0.983) was selected. The optimized extracts were obtained by using a power of 400 W, irradiation time of 5 min and ethanol concentration of 48.467% and again analyzed for total phenolic and flavonoid content. The predicted values of total phenolic and flavonoid content were 3.399 mg GAE/g and 0.600 mg QE/g of the extracts respectively, whereas the observed values were 3.49 mg GAE/g and 0.61 mg QE/g of the extracts. The percentage error was found to be less than ±5%, which is acceptable as shown in Table 3.

Fourier transfer infrared spectroscopy

The FTIR analysis of optimized extract was done to identify the was done to determine the important functional groups present. The FTIR spectrum of the optimized extract is shown in Figure 6.

The most intense broad band observed between 3414 and 3240 cm^{-1} indicates the stretching of a polymeric hydroxyl group (OH), which is representative of polyphenolic compounds.[21] The sharp band at 2920 cm^{-1} and 2850 cm^{-1} corresponds to aromatic CH stretching. The band at 1615-1580 cm^{-1} corresponds to C-H and C=C-C stretching. Phenolic C-O stretching was observed at ~1200 cm^{-1}. This stretching is due to the C-O of pyran, typical of flavonoid C-rings.[22] Therefore, the identified functional groups in

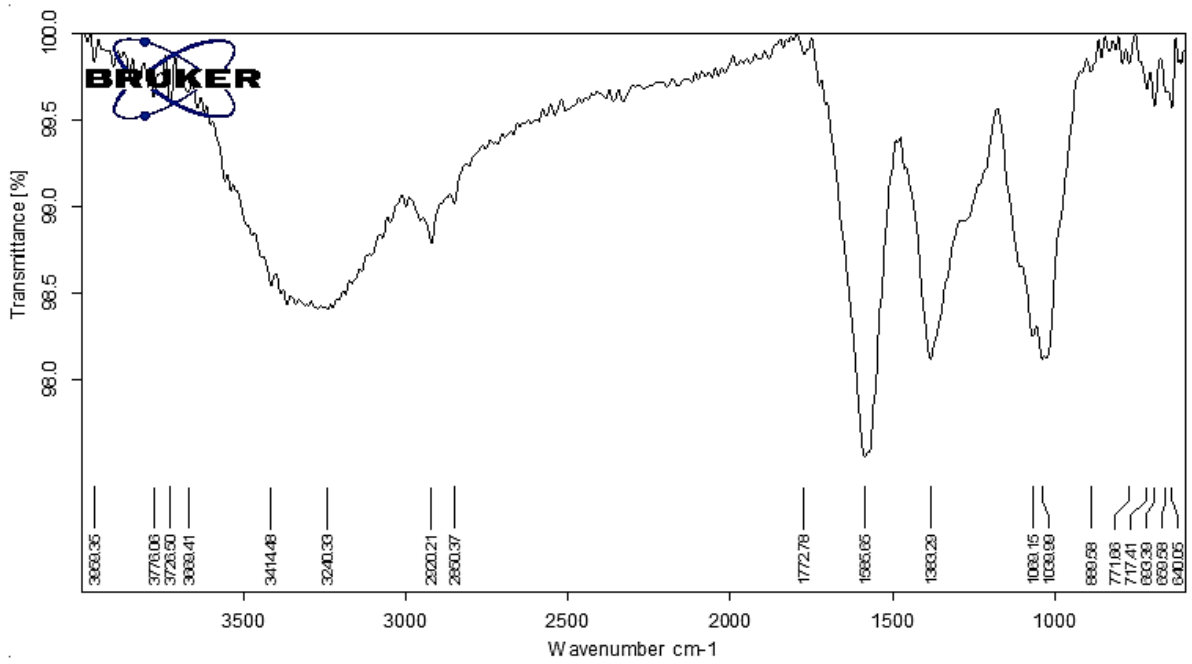

Figure 6: FTIR spectrum of optimized *Dioscorea bulbifera* leaf extract obtained by MAE.

Dioscorea bulbifera leaf extract showed the presence of phenolic compounds.

CONCLUSION

In the current study, the Box Benkhen design was successfully utilized to optimize the parameters of the MAE for improving the yield of total phenolic compounds and total flavonoids from the leaves of *Dioscorea bulbifera*. The microwave power, irradiation time and ethanol concentration have significant effects on the extraction of phenolic compounds. The optimized factors include the power of 400 W, irradiation time of 5 min and ethanol concentration of 48.46% which produced total phenolic content of 3.49 mg GAE/g and total flavonoid content of 0.61 mg QE/g of the extracts. The optimized extract with a higher yield of polyphenolic compounds can be a potential candidate for investigation of antioxidant activity, hence further study can be done to analyze the antioxidant properties by *in vitro* or *in vivo* methods.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MAE: Microwave assisted extraction; **BBD:** Box Benkhen Design; **ANOVA:** Analysis of Variance; **TPC:** Total Phenolic Content; **TFC:** Total Flavonoid content; **GAE:** Gallic Acid Equivalent; **QE:** Quercetin Equivalent; **RSM:** Response Surface Methodology; **FC:** Folin Ciocalteau; **FTIR:** Fourier Transform Infrared Spectroscopy.

SUMMARY

Various studies reported different conventional methods for the extraction of leaves of *Dioscorea bulbifera* for obtaining phenolic compounds. Our study utilized one of the modern methods of extraction, i.e., MAE, to obtain the phenolic-rich extract. The MAE was optimized to find out the suitable extraction condition to obtain better extraction efficiency of phenolic compounds. Since the phenolic compounds are rich sources of antioxidants, optimized MAE condition will be a quicker, economical way of obtaining extracts.

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