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Optimization of Microwave-assisted Extraction Method for the Yield of Extraction and Total Phenol Content from Moringa Leaves (*Moringa oleifera* Lam.) var. PKM 1

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ABSTRACT

Moringa (*Moringa oleifera* Lam.) is an incredible plant with a storehouse of essential nutrients like protein, vitamins, minerals and phytochemicals such as tannins and flavonoids. From the qualitative screening**,** phytochemical was highly present in hydroethanolic extracts. The recovery of these biomolecules could be achieved by advanced novel extraction methods like Microwave-assisted extraction. The main aim of this experimental trial was to optimize with a range of three independent factors including power (500W-700 W), temperature (30-50°C) and extraction time (20-40 min) consisting of two levels of dependent variables like extraction yield (EY) and total phenol content (TPC) using Response Surface Methodology (RSM) constructed by Central Composite Design (CCD) with 20 runs. The optimized extraction method recorded 14.64 to 17.65% of extraction yield and 63.36 to 76.40 mg GA/ gram of total phenol content. As a result of the optimization of microwave-assisted extraction, recovery of biomolecules from the dried moringa leaves was achieved by increasing the extraction temperature with the microwave power of the instrument.

Key words: Moringa, microwave-assisted extraction, biomolecules, response surface methodology, ethanolic extraction

INTRODUCTION

In recent days, the extraction of various biomolecules of human interest are extracted from plant parts as the cheapest and most effective components for the manufacture of various chemical drugs (Alvin *et al.*, 2014). Many secondary metabolites present in the plants have medicinal value and can be tapped by various pharma industries for the manufacture of nutraceutical products that could be consumed on daily basis (Zhao *et al.*, 2019). Regular intake of chemical drugs causes various side effects in human beings and the environment, that can be overcome by extraction of similar phytocomponents from plant sources to cure serious diseases like cancer, hypertension and overweight (Ahmad *et al.*, 2016). Various secondary metabolites that have a wide range of beneficial effects on human beings were found in moringa (*M. oleifera* Lam.) in dried or dehydrated leaves than in fresh leaves (Vázquez-Espinosa *et al.*, 2019).

Moringa is a tree found to be grown all over the world with its native from the sub-Himalayan tract of India, Pakistan, Bangladesh, Afghanistan and later it was distributed to various continents of the world with wide adaptability to all types of soil and stress. Moringa is one of the power-packed nutrient-rich and medicinally important crops

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containing major nutrients like iron, calcium, carotenoids, phosphorus and important amino acids (Rodríguez-Pérez *et al.,* 2016; Castillo-Lopez *et al.*, 2017). All these beneficial phytocomponents from the moringa leaves could be used to cure several diseases and alleviate malnutrition through the extraction process. Direct consumption of moringa leaves shows reduced assimilation of phytonutrients and this can be increased by consuming biomolecules after a suitable extraction method (Ramic *et al.*, 2015). The quality and quantity of the biomolecules are influenced by the extraction methodologies, temperature, time and other various factors (Rodríguez-Perez *et al.*, 2016).

Extraction of biomolecules from the plant source can be recovered through various extraction methods like conventional and non-conventional methods which include, maceration, soxhlet, pressurized Solvent Extraction, Supercritical Fluid Extraction, Ultrasound-Assisted Extraction and Microwave Assisted Extraction (Uysal *et al.*, 2019). During the process of extraction, biomolecules are extracted from the solid plant matrix through the solvent liquid phase by the leaching principle (Geankoplis *et al.*, 2018). The recovery of these phytonutrients can be enhanced by imposing external energy on the plant samples in the form of heat, microwave and ultrasonic waves through novel extraction methods which lack conventional extraction methods (Lasta *et al.*, 2019). The main objective of the experiment was to evaluate the efficiency of microwave-assisted extraction method in the extraction of moringa biomolecules by optimizing the power, temperature and time of extraction concerning the extraction yield and total phenol content in response to the above-mentioned independent factors using Response Surface Methodology (RSM) based on Central Composite Design (CCD). The RSM statistical design was to evaluate and optimize the interaction between the independent and dependent variables.

MATERIALS AND METHODS

The solvents and reagents essential for the extraction process and other biochemical analyses such as 99% pure ethyl alcohol, Folin-Ciocalteu reagent, sodium carbonate, sodium hydroxide, aluminium chloride, methanol, sodium nitrate, potassium acetate and standards like quercetin, ascorbic acid, catechol and gallic acid were purchased from Hi-Media, Merk, Central Drug House and Sigma Aldrich.

Moringa leaves of PKM 1 variety were harvested from the organic field at the western block of Horticultural College and Research Institute, Periyakulam and fresh leaves were cleaned, washed and dried using a solar cabinet dryer at 40±2°C until the leaves crumble. Dried leaves were grounded using a commercial grade hammer mill and sieved using a 250 µm sieve. Pulverized moringa dried leaves were stored in dark and refrigerated conditions for further analysis.

Microwave-assisted extraction was performed using ETHOS™ X equipped with a dual 950 W magnetron of the maximum power of 1900 W manufactured by Milestone, Italy. Extraction of biomolecules from the moringa leaves samples was done by adding 3 g moringa powder with 30 ml of 70% ethanol in high-pressure TFM microwave vessels provided with the equipment. After extraction with RSM-generated differential runs for optimization, the moringa extracts were centrifuged and concentrated using a rotary vacuum evaporator (Equitron Roteva 66 series, Medica Instrument Mfg. Co., Mumbai) and stored at 4 °C for further analysis.

The extraction yield of each experimental run was estimated based on the method described by Cho *et al.* (2020). The mass of the dried moringa extracts after rotary evaporation was calculated and the extraction yield obtained was expressed as percentages using the following equation:

$$\text{Yield (\%)} = \frac{\text{(Weight of the lyophilized extract (g)}}{\text{Weight of the plant sample taken for extraction}} \times 100$$

Total phenolic content in the moringa leaf extract from different runs from the experimental trial was estimated by a spectrophotometric method using a Folin-Ciocalteau reagent with slight modifications as described by Rakesh *et al.* (2021). In brief, dried moringa leaf extracts were re-diluted using deionized water at a concentration of about 1 mg per ml. Using a micropipette 100 µl of the prepared extract was pipetted into the

96 well microplate and further diluted with 400 µl distilled water to the mixture 1:1 diluted FC reagent was added. The microplate containing a mixture of experimental runs was incubated under dark condition for 1 h to develop greenish-blue colour. The absorbance of the extracts was read using a Bio-Rad microplate reader in triplicates at 650 nm. Five hundred ml distilled water with FC reagent without plant extract was considered blank and gallic acid was taken as a standard to estimate the amount of phenol content in the extracts.

The phytochemical profiling of moringa leaf extracts from microwave-assisted extraction (MAE) was performed using Trace GC ultra-chromatograph system (Thermo Fischer Scientific, Austria) coupled to Thermo Scientific DSQ II quadruple MS. The freeze-dried MAE moringa leaf extract from optimized paramteres was resuspended in HPLC grade methanol (99.9% Pure) in the ratio 1 mg/ml of methanol and sonicated using water bath sonicator. The sonicated moringa extracts were filtered using 0.45 µm membrane filter and introduction into the equipment by Tri-plus RSH head space auto sampler. Methanolic extract was made to pass through 5% phenyl methyl silicone fused silica capillary column (TG-SQC, 15 m in length, 0.25 mm I.D., and 0.25 µm film thickness) with ionizing energy of 70 eV and pure helium gas (99.99% pure) as carrier gas (Flow rate: 1 ml/min; Split flow: 10 ml). Column temperature was programmed with 50°C as initial temperature for 1 min and progressively raised to 150°C by 25°C/min rate. Using xcalibur software, spectra pattern and chromatograms were compared with the National Institute of Standards and Technology's library of NIST11.LIB database. unknown phytochemicals present in MAE moringa extracts were identified (NIST library).

The experimental design of the microwave-assisted extraction method was performed from the central composite design by studying the interactions between the independent and dependent variables by RSM (Kaderides *et al.*, 2019). The effect of the independent factors (Table 1) was X_1 power of the instrument (500W to 700W), X_2 extraction temperature (30 to 50°C) and X_3 extraction time (20 to 40 min). Two response variables were taken as dependent factors to represent quantitative (Extraction yield) and qualitative (total phenol content) attributes of MAE from dried moringa leaves. The RSM experimental design was generated by DESIGN EXPERT v13.0 software to develop a quadratic model that fitted the experimental data to draw response D plots and optimization of MAE extraction process.

Table 1. Independent factors and their range set in experimental design

Input variables	Symbol	Levels		
	Xi	-1	0	+1
Power W	X1	500	600	700
Temperature (°C)	X2	30	40	50
Time (Min)	X3	20	30	40

Two levels of response variables were selected and 20 extraction runs were done to predict the optimization process. Estimation of pure error at the sum of square was done in triplicates at the centre of the experiment and the interactions between the dependant and independent variables and their responses were presented in Table 2. From the experimental values, a quadratic model was fitted and regression equations were calculated.

$$Y = \beta_0 \sum_{i=0}^{n} \beta_i X_i + \sum_{i=0}^{n} \beta_{ii} X_i^2 + \sum_{i} \sum_{i=0}^{n} \beta_{ij} X_i X_j + r$$

Where, Y represented the responses such as extraction yield (%), total phenol content (mg GA/g) and constant (β_0). Further, X_i and X_j represented the input independent variables with the number of independent parameters (n) involved in the experiment. The coefficient of the regression model, linear, quadratic and interaction terms were denoted by β_i, β_{ii} and β_{ij}, respectively. The interactive effects of the variables were represented as three-dimensional surface plots by keeping other variables constant and the significance of P 0.05, R square and adjusted R square values were calculated. The value of R^2 should be greater than 0.90 for the best fit of the regression model.

Data analysis of the extraction yield and total phenol content was done using SPSS 27.0 for Windows under a completely randomized design (CRD).

RESULTS AND DISCUSSION

The effect of MAE parameters on the extraction yield of moringa leaf extracts is presented in Table 3. The extraction yield varied from 14.64

Table 2. Optimization of extraction parameters for moringa leaf powder using MAE by RSM

Run	Factors			Extraction yield (%)		Total phenol content (mg GA/g)	
	X_1 (W)	X_2 (°C)	X_3 (Min)	Experimental	Predicted	Experimental	Predicted
1	600	40	30	17.51	17.54	75.73	75.87
2	700	30	20	16.05	16.09	69.45	69.64
3	500	50	40	15.04	15.01	65.1	64.94
4	600	50	30	16.17	16.26	69.97	70.37
5	700	50	20	15.11	15.17	65.4	65.66
6	750	40	30	16.26	16.21	70.35	70.15
7	600	40	30	17.38	17.54	75.16	75.87
8	700	30	40	16.18	16.23	70.01	70.2
9	500	50	20	15.67	15.63	67.81	67.65
10	700	50	40	16.04	15.96	69.39	69.04
11	600	40	30	17.55	17.54	75.9	75.87
12	500	30	40	15.36	15.31	66.48	66.26
13	500	30	20	16.51	16.6	71.41	71.79
14	600	30	30	17.01	16.88	73.55	73.02
15	600	40	40	17.14	17.25	74.12	74.66
16	400	40	30	14.64	14.66	63.36	63.44
17	600	40	20	17.65	17.5	76.4	75.73
18	600	40	30	17.61	17.54	76.25	75.87
19	600	40	30	17.64	17.54	76.31	75.87
20	600	40	30	17.47	17.54	75.63	75.87

Table 3. Regression coefficients and ANOVA estimated for extraction yield of MAE moringa leaf extract

Source	Sum of squares	d. f.	F-value	p-value	
Model	18.42	9	150.64	< 0.0001	Significant
A-Power	0.1688	1	12.42	0.0055	
B-Temperature	0.9486	1	69.82	<0.0001	
C-Time	0.1513	1	11.13	0.0075	
AB	0.0008	1	0.0589	0.8132	
AC	1.01	1	74.20	<0.0001	
BC	0.2178	1	16.03	0.0025	
A^2	7.66	1	563.92	<0.0001	
B^2	2.97	1	218.57	<0.0001	
C^2	0.0814	1	5.99	0.0344	
Residual	0.1359	10			
Lack of fit	0.0905	5	2.00	0.2330	Not significant
Pure error	0.0453	5			
Cor total	18.56	19			
R^2	0.9927				
Adjusted R^2	0.9861				
Predicted R^2	0.9598				

to 17.65% in 20 experimental treatments. The mean values of the extraction yield from the moringa dried leaves were 16.50%. The F value of the model for extraction yield was 150.64, which implied that the model was significant. The extraction yield response of the experimental extraction runs was influenced by A, B, AB, A^2 and B^2. The p values were found to be significant at 0.05 in this condition. On the other hand, the R^2 value of 0.9927 was found to fit with the predicted and actual values of the trial. The predicted R^2 value was 0.9598 stating that the adjusted R^2 value (0.9861) was in logical agreement. The 3D plots of the cumulative effect of extraction temperature and power is presented in Fig. 1. From the results obtained, it was found that at the elevated temperature (50°C) and power (700W) there was a significant decrease in the extraction yield. At the level of moderate temperature (30°C) there was a maximum yield of biomolecules (17.53%) from the moringa leaves at 600W of operation power. These results were similar to the findings of Rodríguez-Perez *et al.* (2016) and Chen *et al.* (2017) in moringa, and Ameer *et al.* (2017) in stevia.

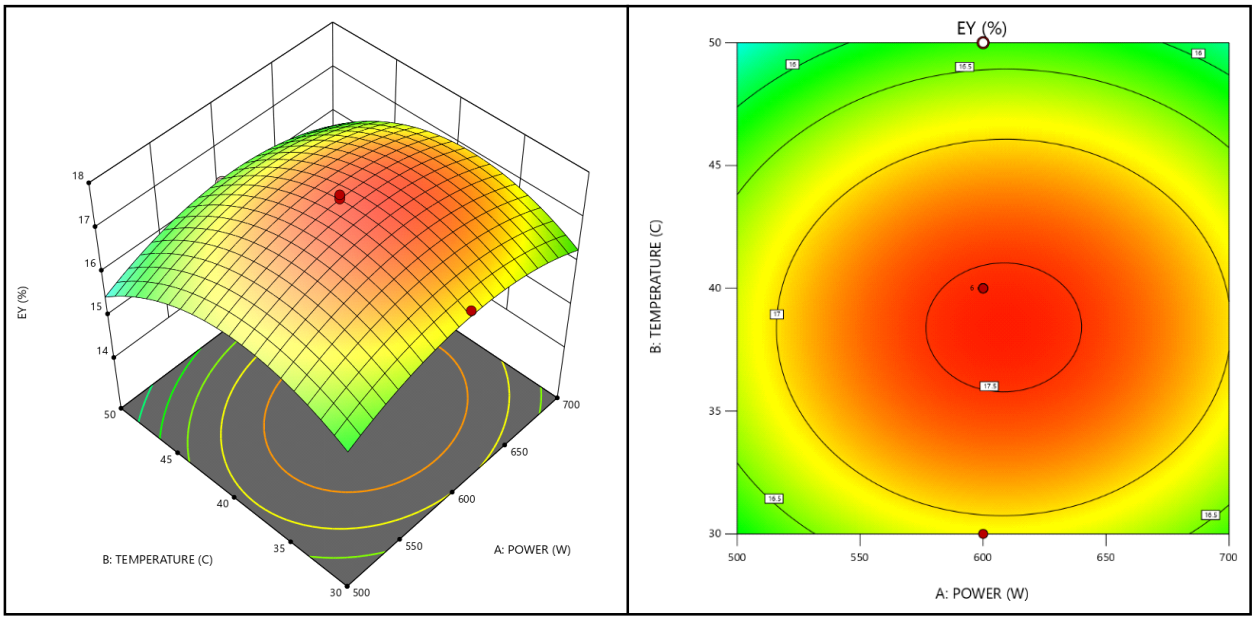

Fig. 1. 3D and 2D plots of extraction yield against power and temperature.

As the extraction temperature and power increased above 50°C and 700W the yield was decreased to the effect of high energy in the form of microwaves produced from the magnetrons in the instrument. At a lower level of temperature (30-40°C) with 500 W-600 W microwave power, extraction yield was recorded higher and it might be due to the increase in the diffusion rate of solvent into the plant matrix which led to the leaching of metabolites into the solvent phase. Due to these reasons, a better extraction yield was obtained from the optimum temperature and power of the experimental runs and biochemical analysis.

The microwave-assisted extraction of moringa dried leaves through hydroethanolic extraction showed a better yield of phenolic compounds. Among the 20 runs, the total phenol content ranged from 63.36 to 76.40 mg GA/g with independent variables ranging in power, temperature and time of extraction. The quantity of total phenol content in each run showed significant differences and the mean values of TPC for the experimental trial were recorded as 71.39 mg GA/g. The model F value of 144.12 with a p-value < 0.0001 showed that the model was significant and also the p values of AC, A^2 and B^2 were less than 0.0001 showing that intercepts were

Table 4. Regression coefficients and ANOVA estimated for total phenol content of MAE moringa leaf extract

Source	Sum of squares	d. f.	F-value	p-value	
Model	343.38	9	144.12	< 0.0001	Significant
A-Power	3.13	1	11.84	0.0063	
B-Temperature	17.5	1	66.12	<0.0001	
C-Time	2.88	1	10.89	0.0080	
AB	0.012	1	0.0454	0.8356	
AC	18.57	1	70.16	<0.0001	
BC	3.99	1	15.07	0.0030	
A^2	142.91	1	539.81	<0.0001	
B^2	55.74	1	210.53	<0.0001	
C^2	1.47	1	5.56	0.0401	
Residual	2.65	10			
Lack of fit	1.74	5	1.91	0.2478	Not significant
Pure error	0.9106	5			
Cor total	346.03	19			
R^2	0.9927				
Adjusted R^2	0.9861				
Predicted R^2	0.9598				

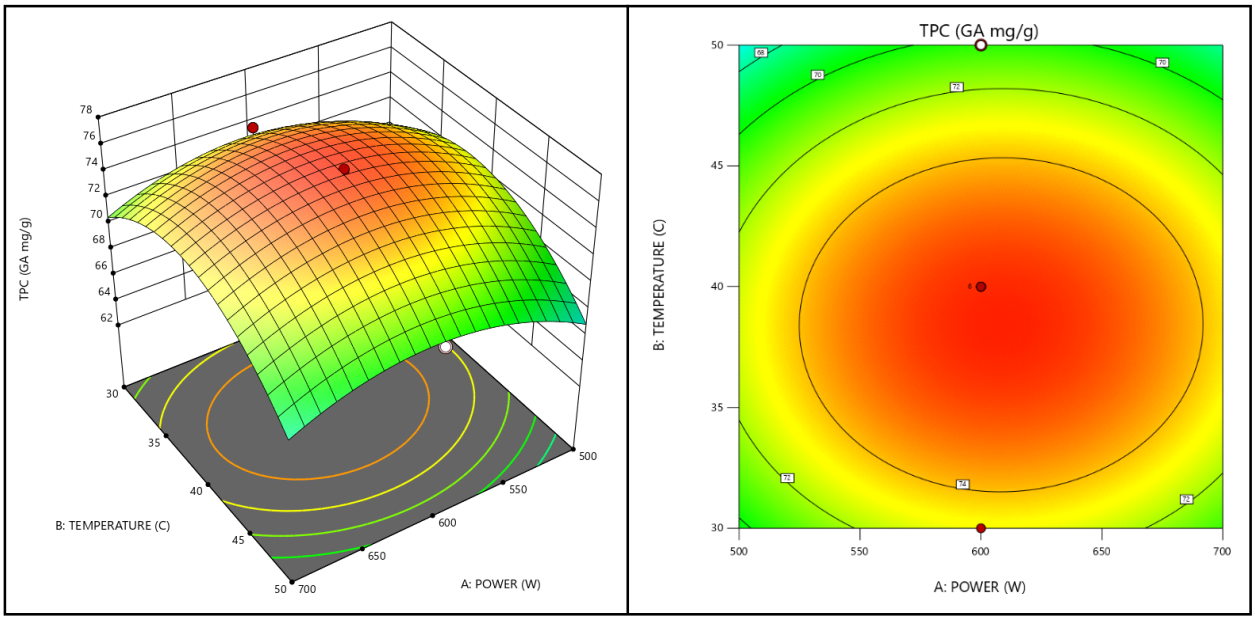

Fig. 2. 3D and 2D plots of total phenol content against power and temperature.

significant (Table 4). The R square value (0.9923) showed that predicted and actual values fitted well. As the predicted R square value was 0.9586, it was in reasonable agreement with the adjusted R square value (0.9855). The influence of response factors from the independent factors of the instrumental parameters was represented in a 3D plot by combining the extraction temperature and power (Fig. 2). Maximum total phenol content of about 76.40 mg GA/g was obtained at optimal power of 600W at 40°C for 30 min of extraction time and also with an increase in temperature and power above 50°C and 700W, respectively, showing a decrease in total phenol content. This might be mainly due to the rise in temperature and the influence of microwave energy in destroying the phenolic compounds as they were temperature sensitive leading to oxidation of phenolic derivatives (Nayak *et al.*, 2015).

Increased yield of phenolic compounds from the moringa leaves might be achieved by rupturing of the plant cells along with solvent diffusion into the matrix caused due to the vibrations of the water molecules present in the liquid solvent phase by the interactions of microwaves. These results concerning the maximized yield of total phenol content were similar to the findings of Dahmoune *et al.* (2015) in *Myrtus communis,* Karami *et al.* (2015) in licorice roots, Rodríguez-Perez *et al.* (2016) and Chen *et al.* (2017) in moringa.

A desirable approach was employed for the maximum recovery of biomolecule yield and phenol content by generating RSM experimental models with optimization of the independent parameters such as power, temperature and duration of extraction. The plot of the experimental responses and predicted values showed no significant deviation between the corresponding values (Fig. 3). The point prediction of the optimized design of the experimental trial showed the highest mean extraction yield (16.50%) and total phenolic compound (71.39 mg GA/g) extraction. These were near the predicted mean values (17.53% and 75.87 mg GA/g) of the trial run with 95% confidence at respective parameters 600W power, 40°C and 30 min of extraction period of microwave-assisted extraction methodology.

The GC-MS analysis of moringa leaf extract from microwave-assisted extraction method resulted in 31 major compounds. The mass spectra of the unknown phytomolecules were characterized using NIST library database. Important phytocompounds detected using GC-MS are listed in Table 5. Based on the previous research outcomes of Majumder *et al.* (2019) it was perceived that microwave-assisted extraction of moringa leaves possessed various medicinal values such as anti-hypercholesterolemia, anti-arthritic, analgesic, anti-anginal, anti-hypertensive, anti-bacterial and anti-fungal properties.

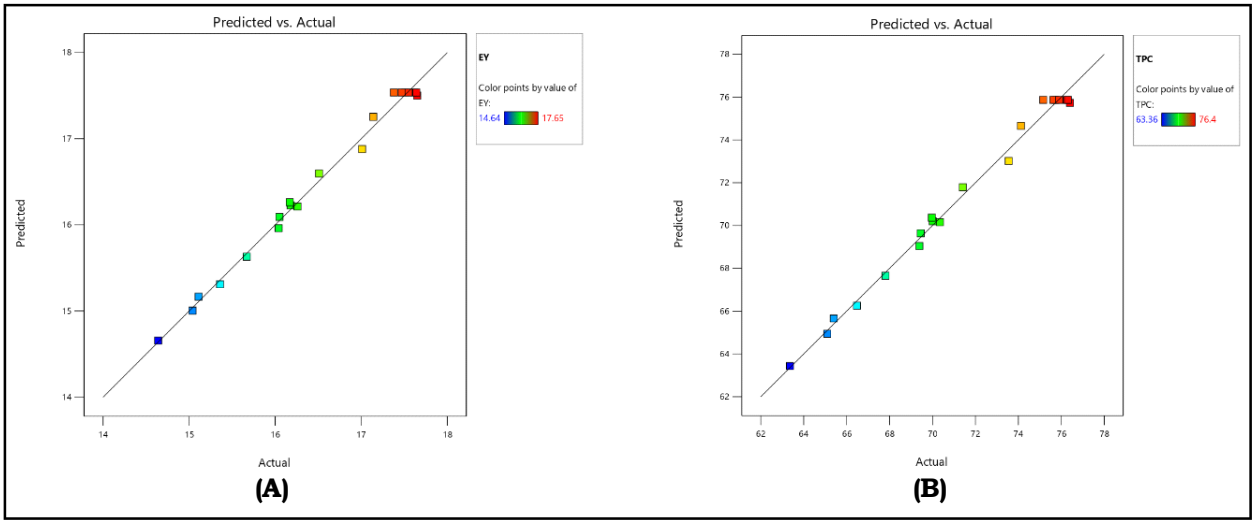

Fig. 3. Linear regression plots of the predicted and experimental values for extraction yield (A) and total phenol content (B).

Table 5. List of identified compounds in moringa leaf extract from microwave-assisted extraction

Name of the compound	Molecular weight (g/mol)	Molecular formula	Retention time (min)	Uses	Reference
Thieno[2,3-c]furan-3-carbonitrile,2-amino-4,6-dihydro-4,4,6,6-tetramethyl-	124.16	C_6H_4OS	2.39	Antibacterial, analgesic, anti-anginal and non-opoid effect	Kadhim *et al.* (2016)
Arsenous acid, tris (trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$	6.79	-	-
2-Methyl-3,5-dinitrobenzyl alcohol, tert-butyldimethylsilyl ether	326.42	$C_{14}H_{22}N_2O_5Si$	34.3	Antimicrobial effect	Abd El-Karim (2016)
Ethyl iso-allocholate	436.31	$C_{26}H_{44}O_5$	35.83	Potent inhibitor for dihydropteroate synthase	Malathi and Ramaiah (2017)
5H-Cyclopropa[3,4]benz[1,2-e] azulen-5-one,9,9a-bis(acetyloxy)-, 7b- trihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-[1aR-(1aà,1bá, 2á,4aá,7aà,7bà,8à,9á,9aà)]-,1,1a, 1b,2,4a,7a,7b,8,9,9a-decahydro-2,4a	406.5	$C_{22}H_{30}O_7$	36.23	Antioxidant and Antibacterial	Majumder *et al.* (2019)
9-Desoxo-9-x-acetoxy-3,8,12-tri-O-acetylingol	536.6	$C_{28}H_{40}O_{10}$	36.91	Anti- inflammatory and antibacterial effect	Shareef *et al.* (2016)
9-Desoxy-9x-chloroingol 3,7,8, 12-tetraacetate	555.1	$C_{28}H_{39}ClO_9$	39.02		
9,12,15-Octadecatrienoic acid,2, 3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-	496.9	$C_{27}H_{52}O_4Si_2$	40.65	Antimicrobial, anti-inflammatory, anti-cancer, anti-oxidant and hyperchol-esterolemia activity	Haber *et al.* (2007) and Good *et al.* (2009)
Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	530.9	$C_{35}H_{62}O_3$	41.67	Antioxidant activity	Neal-Kluever *et al.* (2015)
.psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-	601.0	$C_{42}H_{64}O_2$	43.06	Antioxidant activity	Imhoff and Pfennig (2001)
Propanoic acid,2-(3-acetoxy-4,4, 14-trimethylandrost-8-en-17-yl)-	430.6	$C_{27}H_{42}O_4$	47.18	Anti-microbial and anti-tumour	Kadhim *et al.* (2016)
(22S)-21-Acetoxy-6á,11á-dihydroxy-16à,17à-propylmethylen edioxypregna-1,4-diene-3 ,20-dione	418.5	$C_{24}H_{34}O_6$	47.93	Anti-inflammtory steroid compound to treat eczema and rheumatism	Aroonrerk and Kamkaen (2009)

CONCLUSION

In response to surface methodology, the output in terms of quantitative variables like extraction yield (%) and quantitative variables like total phenol content (mg GA/g) was high at optimized parameters of microwave-assisted extraction with operation power of 600W for 30 min of extraction with 40°C of extraction temperature. The maximum extraction yield was recorded at about 17.65% and total phenol content of 76.40 mg GA/g was obtained at these optimized parameters by RSM. Microwave-assisted extraction was a user-friendly technology that allowing to extraction wide range of secondary metabolites and other biomolecules of medicinal and nutritional value in a very short period. When compared to other conventional methods of extraction, MAE paved the way to extract a wide range of compounds with less utilization of solvents at lower extraction temperatures. These phytocomponents could be further incorporated into human supplementary and could be further fortified in food products to supply all essential phytonutrients through their daily intake. Extracted moringa leaf extract after encapsulation with suitable stable wall materials can be often incorporated in nutritional interventions either as enrichment or also used to develop novel functional food products like an energy bar, energy drink and extruded products (Noodles, pasta). The developed products will be highly suitable for commercialization and alleviating nutritional deficiency problems, especially in vulnerable groups in the community.

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