Received: 25 July 2019 | Revised: 30 December 2019 | Accepted: 25 March 2020

DOI: 10.1111/jfpp.14482

ORIGINAL ARTICLE

Journal of Food Processing and Preservation | Institute of Food Science + Technology ifst | WILEY

Optimising extraction of antioxidants from roasted *Moringa oleifera* Lam. leaves using response surface methodology

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Funding information
TWAS-CSIR postgraduate fellowship program, Grant/Award Number: FR number: 3240280447

Abstract

Optimal conditions for alcoholic extraction of antioxidants from roasted leaves of *Moringa oleifera* Lam. were determined using response surface methodology and the phenolic profile of optimised extract determined by HPLC-PDA. The experimental results adequately fitted the second-order polynomial models as indicated by regression coefficient (.90–.95), lack-of-fit >.05, and absolute deviation .01–.04). Using multiple responses optimization and desirability function, the optimal conditions needed to maximize simultaneously antioxidant activity, phenolic content and extract yield were 65%–75% ethanol, 55–65°C and 40 min. The experimental values were not significantly different ($p > .05$) from those predicted, indicating the suitability of the models in the studied range. HPLC analysis of the extract revealed the presence of quercetin, isoquercetin, caffeic acid, and rutin. Rutin (45 mg/g), a flavonoid, was the major phenolic. The extract exhibited significant antioxidant potential IC_{50} 2,2-diphenyl-2-picryl hydrazyl 1.28 mg/L, ferric reducing antioxidant power 236 mg AAE/g, total phenolic content 176 mg GAE/g and could be used as nutraceuticals.

Practical applications

Moringa oleifera L. leaves were roasted to improve antioxidant function and optimum conditions for the preparation of extract with optimal antioxidant potential determined using the multiple response optimization procedure. The resulting extract was freeze-dried and its phenolic profile characterized by HPLC-PDA. The powdered extract has good antioxidant (radical scavenging and ferric reducing) potential and contained rutin as the major phenolic flavonoid followed by quercetin, isoquercetin caffeic acid, and vanillin. The extract has potential for use in nutraceutical formulations for the prevention and management of oxidative stress and chronic diseases (diabetes, cardiovascular diseases, cancer).

1 | INTRODUCTION

Moringa oleifera Lam. is an edible plant that recently became popular as a dietary supplement and herbal medicine for the management of several chronic diseases such as diabetes (Azad et al., 2017; Fombang & Saa, 2016a), inflammation (Galuppo et al., 2014), hypertension (Fombang, Bouba, & Ngaroua, 2016; Sana, Saleem, & Faizi, 2015), and neurological disorders (Hannan et al., 2014). Majority of its medicinal properties have been attributed to the presence of phenolic compounds particularly flavonoids that show strong antioxidant activity (AOA) (Gopalakrishnan, Doriya, & Kumar, 2016). *M. oleifera* leaves are endowed with high phenolic content and high antioxidant capacity ranging from radical scavenging activity to reducing power and metal chelation (Fombang & Saa, 2016b; Nobosse, Fombang, & Mbofung, 2017). Meanwhile, the antioxidant activity of *M. oleifera* leaves which depends on both concentration and chemical structure

of phytochemicals is affected by several post-harvest treatments such as blanching, drying, (Moyo, Mavumengwana, & Kayitesi, 2017; Nobosse et al., 2017; Potisate, Phoungchandang, & Kerr, 2014), and roasting (Parwani, Bohra, Gupta, & Kumar, 2016). Previous results in our laboratory had shown that roasting treatment significantly improved AOA of *M. oleifera* leaves compared to other treatments such as blanching, drying, and fermentation, in an optimised experiment (unpublished data). Similarly, roasting was found to improve AOA and phenolic content in onions and pepper (Juániz et al., 2016). In addition to post-harvest treatment, the concentration as well as activity of antioxidant components in extracts depends on extraction parameters, such as solvent type and concentration, time, and temperature (Fombang & Saa, 2016b; Gunathilake et al., 2019; Rodríguez-Pérez, Quirantes-Piné, Fernández-Gutiérrez, & Segura-Carretero, 2015). With regards to *M. oleifera* leaves, Nobossé, Fombang, and Mbofung (2018) had reported that methanolic and ethanolic extracts of 45-day-old Moringa leaves exhibited best antioxidant potential. However, in order to maximize the effects of post-harvest treatment, on AOA of *M. oleifera* leaves, it is important to understand the interactions between extraction parameters and to optimize them.

Several investigations have focused on maximizing the extraction of phenolic compounds from Moringa leaves using the "one-factor-at-time" approach (Rodríguez-Pérez et al., 2015; Vongsak, Sithisarn, Mangmool, et al., 2013). The main limitations of this approach are the negligence of interactions between different factors and difficulty to have an optimum involving simultaneously several independent and dependent variables. To overcome these issues, response surface methodology (RSM) is a useful tool for the effective and simultaneous analysis of the effects of several factors and interactions on one or more parameters. It is useful to interpret combined effects of process parameters and to optimize processes involving several independent variables (Kwon, Bélanger, & Paré, 2003). RSM has been applied to determine optimal solid-liquid ratio, temperature, and time for extraction of phenolic compounds, flavonoid, and tannins in Moringa leaf powder (Fombang & Saa, 2016b). Likewise, Premi and Sharma (2016) found optimal ethanol concentration, temperature, time, and particle size leading to maximum phenolic content, flavonoid, and AOA in Moringa seeds using RSM. To obtain maximal AOA from roasted *M. oleifera* leaf extract, the present study was undertaken to determine optimal ethanol concentration, temperature, and time for the production of extracts endowed with highest 2,2-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity, ferric reducing antioxidant power (FRAP), total phenolic content (TPC), and extract yield using RSM and to determine the phenolic composition of the optimized extract.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Standards phenolics (gallic acid, tannic acid, and Vanillin), flavonoids (Catechin, Epicatechin, Kaempferol, Quercetin, isoquercetin, Apigenin, Naringenin, and Rutin), β-carotene, α-tocopherol, ammonium molybdate, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, DPPH, and potassium persulfate (di-potassium peroxydisulfate) were purchased from Sigma-Aldrich (St Louis, USA). Folin Ciocalteu reagent, ethanol reagent grade, Methanol HPLC grade, DMSO reagent grade, HCl, Na_2HPO_4, and sodium carbonate were purchased from Merck (Darmstadt, Germany).

2.2 | Collection and processing of Moringa leaves

Fresh leaves of *M. oleifera* were harvested at 45 ± 2 days after trees were pruned in our experimental garden in Ngaoundéré, Adamawa Region, Cameroon; in accordance with previous studies that showed leaves at this age had highest AOA (Nobossé, Fombang, & Mbofung, 2018). Harvested leaves were immediately transported to the laboratory, cleaned, and spread on stainless steel trays (1 g/20 cm^2) in a torrefier (BCM TORRE PICENARDI (CR) Italy, Model Panacea 2430) and were roasted at 145 ± 5°C for 25 min. These conditions had been defined previously in an optimization experiment (Unpublished data). The roasted leaves were ground using a blender (Moulinex Model LM242027) and sieved to obtain the roasted leaf powder with particles size less than 500μm. The powdered sample was packaged in polyethylene bags and stored at −20°C for further analysis.

2.3 | Extraction procedure

The roasted Moringa leaf powder was extracted by maceration using a magnetic stirrer (IKA C-MAG HS 4, Darmstadt, Germany) set at 3,000 rpm. Three grams (3 g) of the roasted leaf powder was weighed into a 500 ml conical flask and placed in a thermostatic water bath. Extraction was carried out with varying concentrations of aqueous ethanol at temperatures and times indicated by the Doehlert experimental design (Tables 1 and 2) with the solid–liquid ratio fixed at (1/40, w(g)/v(ml)) based on preliminary experiments. For extraction at higher temperatures (>70°C), a condenser was mounted to avoid ethanol evaporation. The resultant extracts were filtered using filter paper (Whatman No.1), concentrated under reduced pressure at 40°C and freeze-dried. Extraction yield, DPPH free radical scavenging activity, FRAP, and TPC of the dried extracts were determined.

TABLE 1 Experimental domain and independent variables for optimization

Factors (symbol, units)	Lower level	Middle level	Upper level
Temperature (X_1, °C)	25	58	90
Time (X_2, min)	20	40	60
Ethanol concentration (X_3, %)	50	72.5	95

TABLE 2 Antioxidant activity of *M. oleifera* leaf extract in the Doehlert experimental design

Runs	X_1 (°C)	X_2 (Minutes)	X_3 (%)	DPPH IC_{50} (μg/mL)	FRAP (mg AAE/g)	TPC (mg GAE/g)	YIELD (%)
1	57.5	40.0	72.5	1,260	210	166	33.26
2	90.0	40.0	72.5	1,512	188	158	31.28
3	25.0	40.0	72.5	1,374	192	170	27.51
4	73.7	57.3	72.5	1,414	175	161	34.55
5	73.7	22.6	72.5	1,541	192	164	33.3
6	57.5	40.0	72.5	1,223	215	169	32.98
7	41.2	22.6	72.5	1,403	190	163	29.9
8	41.2	57.3	72.5	1,406	197	164	29.41
9	41.2	45.7	90.8	2,153	156	144	16.66
10	41.2	34.2	54.1	1,701	190	159	33.35
11	73.7	45.7	90.8	1,960	175	149	25.18
12	57.5	40.0	72.5	1,297	216	171	32.6
13	73.7	34.2	54.1	1,691	181	164	34.65
14	57.5	51.5	90.8	1,681	186	144	28.86
15	57.5	28.4	54.1	1,592	182	161	34.44

Note: Response values are mean values of triplicate determinations. X_1, temperature; X_2, time; X_3, ethanol concentration.
Abbreviations: AAE: ascorbic acid equivalent; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; TPC: total phenolic content.

2.4 | Experimental design and modeling

The experimental domain showing lower, middle, and upper levels of factors considered for optimization is shown in Table 1 and was selected based on preliminary studies using FRAP and DPPH activity. Both FRAP and DPPH did not change significantly with extraction temperature in the range of 25–95°C but increased with extraction time to reach maximum values at 30 min of extraction. While maximum FRAP was obtained with pure water and decreased slightly remaining constant in the range 40%–80%, DPPH activity increased with ethanol concentration to a maximum of 80%. RSM was used for optimization through a Doehlert experimental matrix (Table 2) consisting of 15 experimental runs including three replicates at the center point. This experimental design consisted of three factors namely, temperature (X_1, °C), time (X_2, min), and ethanol concentration (X_3, % v/v). The order of experiments was randomized to minimize the effects of non-controlled factors. The dependent variables were extract yield, DPPH free radical scavenging activity, FRAP, and TPC in dried extracts. Experimental data were fitted to a second-order polynomial model (Equation 1) and regression coefficients obtained.

$$Y = b_0 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_1X_1 + b_2X_2 + b_3X_3 \quad (1)$$

where Y is the dependent variable; b_0, b_1, b_2, b_3, b_{11}, b_{12}, b_{13}, b_{22}, b_{23}, and b_{33} are regression coefficients; and X_1, X_2, and X_3 are the independent variables.

2.5 | Validation and verification of the generated models

The validity of models was checked using statistical tools such as lack-of-fit, regression coefficient, absolute analysis of deviation (AAD) bias factor (Bf), and accuracy factor (Af). The validated second-order polynomial models were used in the response surface analysis software (Stat graphic Centurion XV, Virginia, USA) to generate response surfaces and contour plots necessary in the determination of the optimal area of extraction. Furthermore, the practical verification of the models was done by comparing experimental and predicted values at a selected point in the optimal area.

2.6 | Determination of dependent variables

2.6.1 | Sample preparation

A stock solution of each extract (5 mg/ml) was prepared by dissolving the indicated mass of extract in methanol. Next, serial dilutions were prepared from the stock solution to make solutions of 0.1, 0.25, 0.5, 1, 2.5, and 5 mg/ml which were used in antioxidant activity assays. Ascorbic acid (.01–.1 mg/ml) was used as standard.

2.6.2 | Total phenolic content

TPC of the extracts was determined as reported previously (Nobosse et al., 2017) using the Folin–Ciocalteu's phenol reagent, with gallic

acid as standard. TPC was expressed as gram gallic acid equivalents (GAE)/g of extract.

2.7 | Antioxidant properties of *M. oleifera* leaves

2.7.1 | DPPH scavenging activity

The DPPH scavenging activity was measured as described by Brand-Williams, Cuvelier, and Berset (1995) with slight modifications (Nobosse et al., 2017). The DPPH scavenging activity (%) was plotted against extract concentration and the IC_{50} value (mg/ml) of each extract calculated. The calculated IC_{50} values denote the concentration of the sample required to scavenge 50% of free radicals.

2.7.2 | FRAP assay

The antioxidant potential of *M. oleifera* leaf extracts was determined by their ability to reduce iron using the ferrous ion reducing capacity method as described by Yen and Chen (1995) with modifications reported in Nobosse et al. (2017). Ascorbic acid was used as a reference.

2.7.3 | Extraction yield

The extraction yield (%) was calculated as the ratio of mass of dried extract on mass of sample.

2.8 | HPLC quantification of phytochemicals in extract

Phenolic compounds in the extract obtained under optimized conditions were profiled using HPLC-PDA. The powder extract sample was mixed with methanol at a concentration of 1 mg/ml. The solution was then dried with anhydrous sodium sulphate and filtered through a 0.22 µm nylon membrane filter (Axiva). The analysis was performed on a HPLC system (Shimadzu, Japan) with a LC-20AT HPLC pump, DGU-20A5 degasser, SIL-20A auto sampler and SPD-M20A PDA detector. A Bridge RP18 (Waters, UK) analytical column (5 µm, 250 × 4.6 mm) was used with 20 µl injection volume. The column oven temperature was set at 30°C. The PDA acquisition wavelength was set in the range of 250–400 nm. The analog output channel A at wavelength 280 nm and analog output channel B at 360 nm both with bandwidth 2 nm. The gradient elution was carried out using a water–acetic acid mixture, 97:3 v/v, pH = 2.3 (solvent A) and 100% methanol (solvent B), with a flow rate of 1 ml/min (Zuo, Chen, & Deng, 2002). The binary gradient elution system started at 0% solvent B for 1 min, then 2%–15% for 6 min, 15%–50% for 13 min, 50%–60% for 10 min, 60%–80% for 12 min, 80%–100% for 13 min, and 100%–0% B for 5 min for a total run time of 60 min. The column was equilibrated with 100% phase A for 5 min prior to the next sample injection. Identification of sample' polyphenols was based on the comparison of their retention time and UV absorption spectra with external standards. The quantification of phenolic compounds in the extract was carried out based on the calibration curves obtained with varying concentrations of the reference external standards.

2.9 | Statistical analysis

All the assays were performed in triplicate and results are expressed as Mean ± *SD*. RSM was used for the determination of optimal conditions. ANOVA and Duncan test at a 5% confidence level (1% in the case of yield) were used to test the statistical significance of studied independent variables on individual dependent variables. Sigma Plot 11 (SYSTAT Software San Jose, CA) was used for plotting graphs. Using Stat graphic Centurion XV, Virginia, USA, multiple responses were computed into desirability function which is a measure of how close the fitted value is to the desired value. The difference between the predicted and experimental values was assessed by determining the coefficient of variation.

3 | RESULTS AND DISCUSSION

3.1 | Modeling and optimization of extraction conditions

The optimal extraction conditions for the phenolic content, AOA, and extraction yield were determined using RSM. Table 2 shows the experimental conditions for each dependent variable and the corresponding responses.

3.1.1 | Fitting the model

The experimental data were further analyzed using RSM and second-order polynomial models were generated to correlate the dependent variables to independent variables. These models are useful in predicting the behavior of each dependent variable as a function of the independent process parameters considered in the study. The regression coefficients of individual linear, quadratic and interaction terms were determined (Table 3), and the P-values from ANOVA were used to check the significance of each of the coefficients. To ensure that the selected quadratic models fit adequately the obtained AOA, phenolic content and extract yield in the studied range, the predicted models were tested for adequacy and validity. The fitted models showed a satisfactory coefficient of determination R^2 that varied from 0.90 to 0.95, suggesting a good fit. The lack-of-fit was not significant for extract yield ($p > .01$), DPPH, FRAP and TPC

TABLE 3 Regression coefficients of predicted second order polynomial models

	DPPH ($IC_{50,}$ μg/mL)					FRAP (mg AAE/g)					TPC (mg GAE/g)					Yield (%)				
Coefficient	DF	Coef	SS	F	P	DF	Coef	SS	F	P	DF	Coef	SS	F	P	DF	Coef	SS	F	P
b_0		1,259					213.6					168.7					32.9			
b_1	1	27.4	3,000	2.20	0.27	1	−2.25	20.2	1.96	0.29	1	−2.16	18.78	2.33	0.26	1	3.23	41.9	382.0	**0.002**
b_2	1	−51.4	8,227	6.04	0.13	1	−1.3	5.2	0.51	0.55	1	−0.84	2.21	0.27	0.65	1	0.67	1.42	12.96	0.070
b_3	1	189.5	111,718	82.1	**0.01**	1	−6.7	141	13.6	0.06	1	−9.36	272.7	33.8	**0.02**	1	−6.80	144	1,310	**0.000**
b_{12}	1	−75.0	40,405	29.7	**0.03**	1	−13.8	672	13.9	**0.01**	1	−4.58	25.2	3.13	0.22	1	−3.55	15.1	137.9	**0.007**
b_{13}	1	−85.4	4,227	3.11	0.22	1	22.0	144	28.2	0.06	1	−2.14	3.45	0.43	0.57	1	1.00	0.75	6.90	0.12
b_{23}	1	−1014	4,371	3.21	0.21	1	38.1	291	9.4	**0.03**	1	0.60	0.22	0.03	0.88	1	4.07	9.93	90.48	0.010
b_{11}	1	183.5	39,223	28.82	**0.03**	1	−23.6	790	65.0	**0.01**	1	−6.31	47.85	5.95	0.13	1	−0.36	0.15	1.40	0.36
b_{22}	1	180.8	68,730	50.5	**0.02**	1	−25.6	97.2	76.5	0.09	1	−5.25	1.84	0.23	0.67	1	14.3	13.8	125.8	**0.007**
b_{33}	1	1,192	348,998	256	**0.00**	1	−58.7	845	81.8	**0.01**	1	−17.49	75.04	9.32	0.09	1	−11.9	34.9	318.5	**0.003**
Lack-off-fit	3		42,440	10.4	0.09	3		324	10.4	0.08	3		82.70	3.42	0.23	3		24.9	75.92	0.013
Pure error	2		2,722			2		20.6			2		16.1			2		0.21		
Total corr.	14		941,835			14		3,527			14		1,043			14		317		

Note: X_1, temperature; X_2, time; X_3, ethanol concentration.

Abbreviations: Coef, regression coefficient; DF, degree of freedom; DPPH, 2,2-diphényl 1-picrylhydrazyl; F, *F*-value; FRAP, ferric reducing antioxidant power; P, probability; SS, sum of square; TPC, total phenolic content. Values in red are significant at $p<0.05$.

(p > .05), strengthening the reliability of the models. Moreover, the AAD close to zero indicated little deviation between experimental and fitted values while Af (1.01–1.03) and Bf (0.99–1.0) were close to their respective reference values of one. Hence, all the fitted second-order polynomial models are valid and can be used to define the experimental data in the range considered in this study.

Response surface plots were used for a graphic representation of the individual effect of process parameters as well as their interaction on the dependent variable. Among the three factors considered in the present study, solvent concentration had the most statistically significant effect (p < .05) on all responses (Table 3). This effect is positive for DPPH and negative for FRAP, TPC, and the extract yield.

3.2 | Effect of individual factors and interactions on TPC and AOA of the extracts

3.2.1 | Total phenolic content

Ethanol concentration was the only variable that significantly affected TPC (Table 3, Figure 1). TPC increased with ethanol concentration up to a maximum at 65%–75% and thereafter decreased. The effect of temperature on TPC was dependent on ethanol concentration. Hence, at ethanol concentration between 50% and 75%, total phenolic concentration increased with temperature up to a maximum of 170 mg GAE/g extract corresponding to the temperature range 50–65°C. At higher ethanol concentration, no effect of temperature

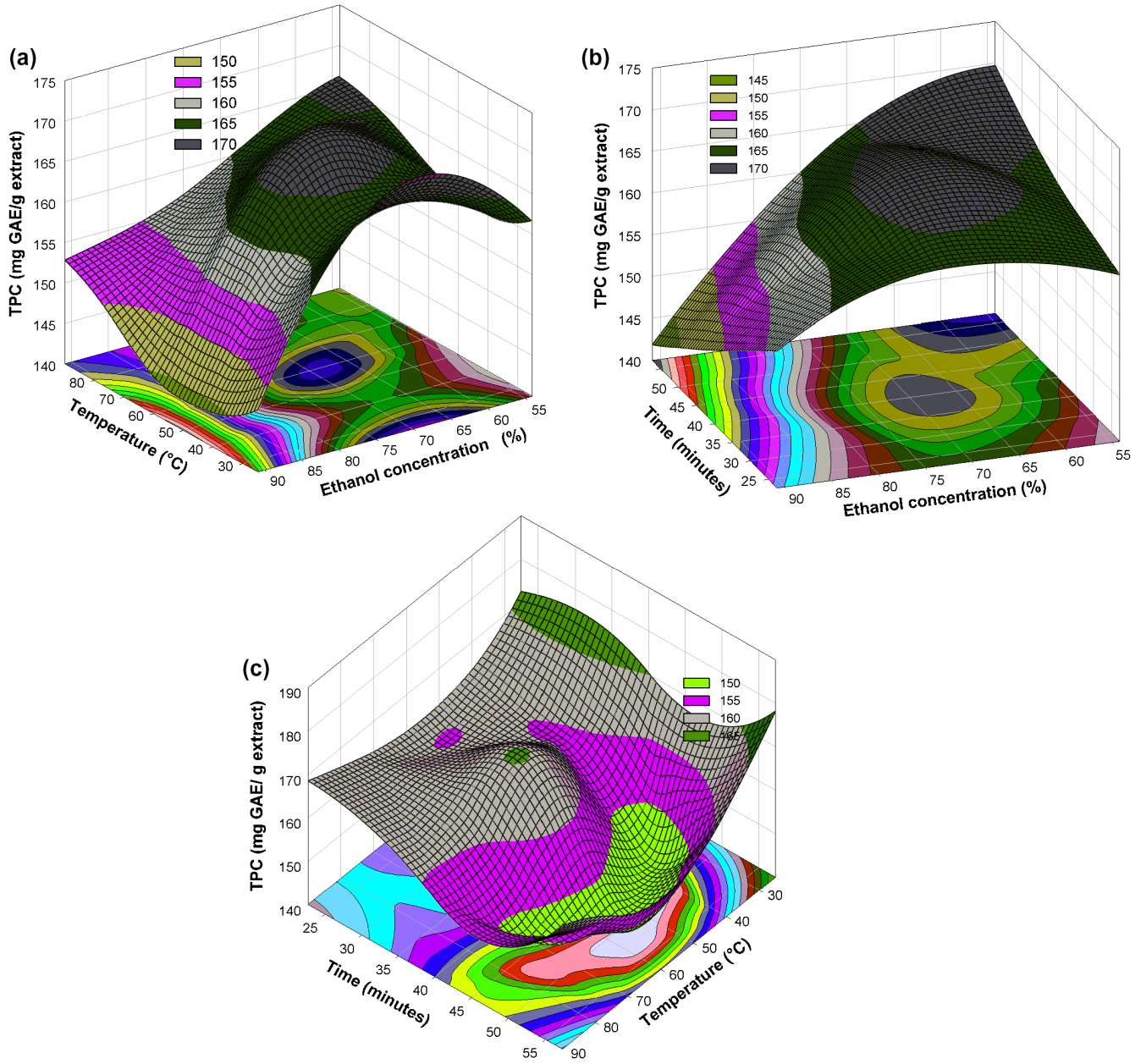

FIGURE 1 Effects of extraction temperature, time, and ethanol concentration on the total phenolic content (TPC) in roasted *M. oleifera* leaf extracts

on phenolic content was observed (Figure 1a). Ethanol concentration of 65%–75% and temperature of 50–65°C were optimal for TPC extraction. Figure 1b depicts the combined effect of time and ethanol concentration on TPC. This interaction was, however, not significant ($p > .05$). Maximum TPC was obtained at an ethanol concentration of 65%–75% and time of 35–45 min. Higher ethanol concentration (>75%, v/v) yielded lower TPC, in accordance with previous reports (Premi & Sharma, 2016). The combined effect of extraction time and temperature on TPC (Figure 1c) shows that at extraction time around 35–40 min, a maximum peak of TPC is observed corresponding to a temperature of 55–60°C. In summary, maximum TPC from roasted leaves of *M. oleifera* is obtained at 50–65°C in 65%–75% ethanol for 35–45 min.

This result is consistent with that of Vongsak, Sithisarn, Mangmool, et al., (2013) who found that maceration of dry Moringa leaves with 70% ethanol ensured maximum extraction of total phenolics. The behavior of total polyphenolic compounds (TPC) with ethanol concentration will depend on the polyphenol's structure and solvent polarity. Polyphenols are organic molecules with a great affinity for organic solvents. They equally contain polar functional groups (hydroxyl groups, carbonyl groups), therefore, addition of water to ethanol will increase solvent polarity and improve the extractability of hydrophilic phenolic components (Spigno, Tramelli, & Faveri, 2007). However, at higher concentrations of ethanol with reduced polarity extraction of some of these polar groups may be inhibited thus accounting for the subsequent reduction in polyphenol content (Huaman-Castilla et al., 2019). Several studies have reported solvent concentration as the most important factor contributing to the extraction of soluble solids from various natural products (Kwon et al., 2003; Liyana-Pathirana & Shahidi, 2005; Vongsak, Sithisarn, Mangmool, et al., 2013) which is consistent with our findings.

The increase in TPC with initial increase in extraction temperature could be explained by enhanced solvent penetration in the plant which improves both solubility and diffusion rate of polyphenols; but at higher temperatures, thermal degradation of phenolic compounds can occur which explains the decline in TPC beyond 70°C (Spigno & De Faveri, 2007). Mild heating may soften plant tissues, hydrolyze the phenol–polysaccharide or phenol–protein bonds and weaken cell wall integrity, thereby enhancing phenolics solubility (Spigno et al., 2007).

3.2.2 | Effect of process variables on antioxidant activity of extracts

DPPH scavenging activity

Ethanol concentration, the interaction between extraction temperature and time as well as the quadratic term of every factor significantly ($p < .05$) affected DPPH scavenging activity (Table 3). Figure 2 presents the combined effects of these variables on DPPH scavenging activity of *M. oleifera* roasted leaf extract. DPPH scavenging activity increased with ethanol concentration up to a maximum value (IC_{50} = 1,300–1,400 μg/ml) at 65%–75%, and then decreased. Likewise, in the same range of ethanol concentration, DPPH increased with extraction temperature to a maximum of 55–65°C then decreased (Figure 2a). Moreover, DPPH scavenging activity peaked at 30–55 min for ethanol concentration between 65% and 75% (Figure 2b). Extraction time between 35 and 45 min, at 50–60°C gave best DPPH activity. Outside this range, DPPH activity was minimal. Maximum DPPH activity represented by the lowest value of IC_{50} was obtained in the range 50–60°C in 65%–75% ethanol for 30–45 min. In these optimal conditions, DPPH activity of roasted Moringa leaf extract was higher than that reported in dried Moringa leaves by Nouman et al. (2016) and Vongsak, Sithisarn, Mangmool, et al. (2013).

Effect of process variables on FRAP

The ANOVA test (Table 3) shows that none of the three factors taken individually significantly affected FRAP in roasted leaves of *M. oleifera*. Nevertheless, interactions between temperature and time, ethanol concentration and time as well as the quadratic effect of ethanol concentration and temperature had a significant influence on FRAP ($p < .05$). Figure 3 presents the response surfaces showing the combined effects of process variables on FRAP in roasted Moringa leaf extract. At lower ethanol concentration, FRAP was not influenced by both extraction temperature and time, while a positive effect of these process parameters was observed at higher ethanol concentration, suggesting that the effects of time and temperature are concentration dependent. FRAP increased with ethanol concentration up to a maximum value of 210–220 mg AAE per gram of the extract corresponding to 65%–75% ethanol concentration for an extraction temperature of 50–60°C and time of 30–45 min (Figure 3a,b). Similar to the trend observed for TPC and DPPH, maximum FRAP was recorded at 50–60°C for 35–45 min (Figure 3c).

It is worth noting that polyphenol content and antioxidant activity (DPPH radical scavenging activity and FRAP) varied with ethanol concentration, extraction temperature, and time. However, both maximum antioxidant activity and maximum polyphenol content in *M. oleifera* roasted leaf extract were obtained in the same optimal range. Hence, supporting the fact that polyphenols are accountable for antioxidant activity in roasted leaf extract of Moringa in accordance with previous reports (Nobosse et al., 2017; Premi & Sharma, 2016).

3.3 | Effect of process variables on extract yield

Extract yield was significantly ($p < .05$) influenced by both ethanol concentration and temperature, as well as their quadratic effects and the interaction between extraction time and temperature (Figure 4). Extract yield decreased with increase in ethanol concentration but increased with extraction temperature. The maximum extract yield of 35% was recorded in the range of 65%–75% ethanol concentration for an extraction temperature of 50°C and higher (Figure 4a). In the same range of ethanol concentration,

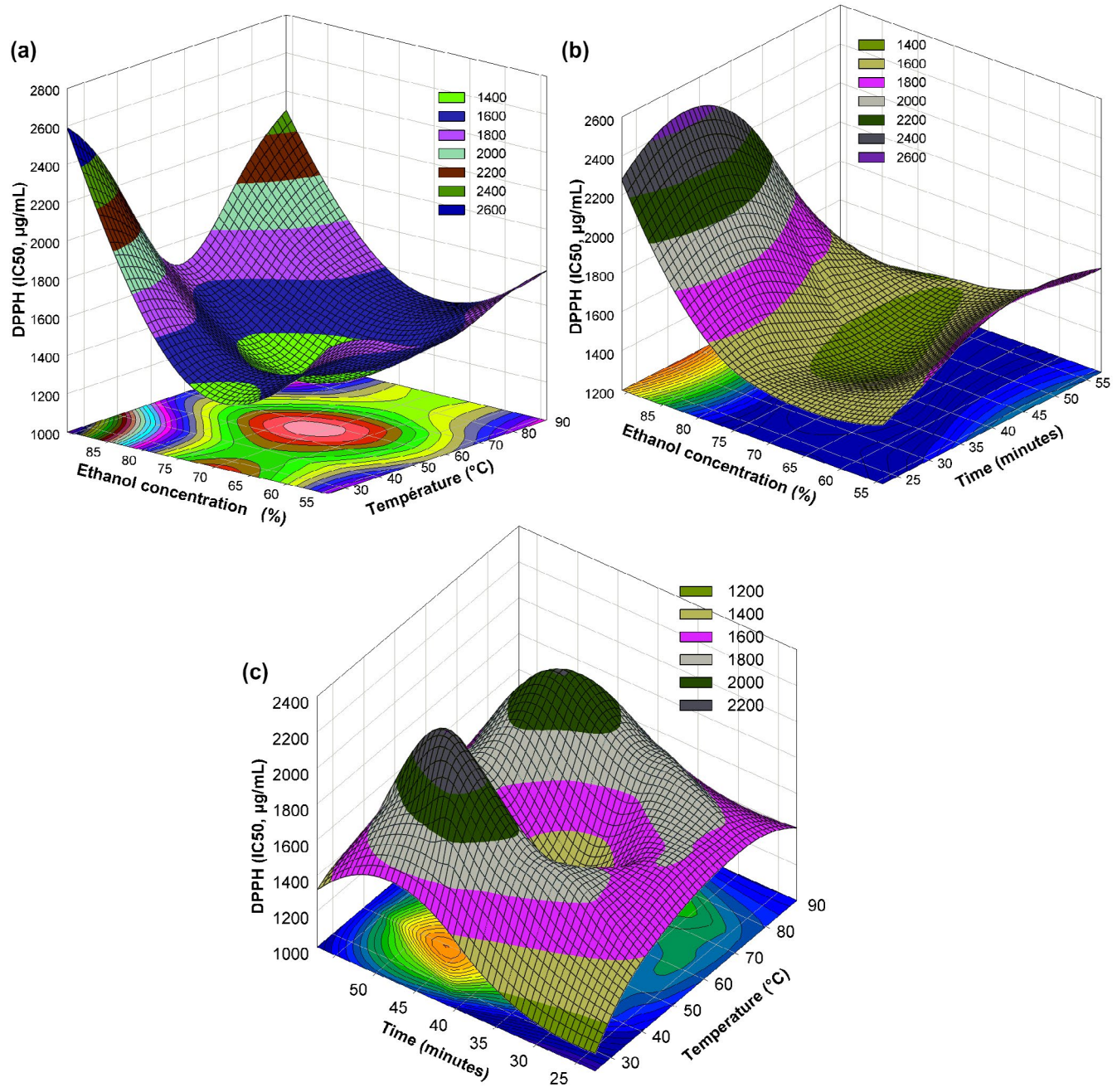

FIGURE 2 Effects of extraction temperature, time, and ethanol concentration on the DPPH scavenging activity in roasted *M. oleifera* leaf extracts

the maximum extract yield was obtained between 45 and 55 min (Figure 4b). The combined effect of extraction time and temperature on extract yield reveals that the maximum (35%) is obtained in the range 20–40 min independently of temperature and could attain 40% (Figure 4c). In summary, the maximum extract yield was obtained when *M. oleifera* leaves were extracted at a temperature higher than or equal to 50°C in ethanol concentration of 65%–75% for 20–45 min. A similar extraction yield has been reported from maceration of dried leaves of Moringa in 70% ethanol (Vongsak, Sithisarn, Mangmool, et al., 2013).

3.4 | Optimization

After validation of the second-order polynomial model relating the responses to the independent variables studied, the optimization procedure was performed to determine the levels of the factors where all dependent variables are simultaneously optimal.

For this study, the criteria settings were to find conditions that maximize DPPH scavenging activity, FRAP, TPC, and extract yield. The multiple response optimization procedure was used to determine the optimal operating conditions in which all these criteria are

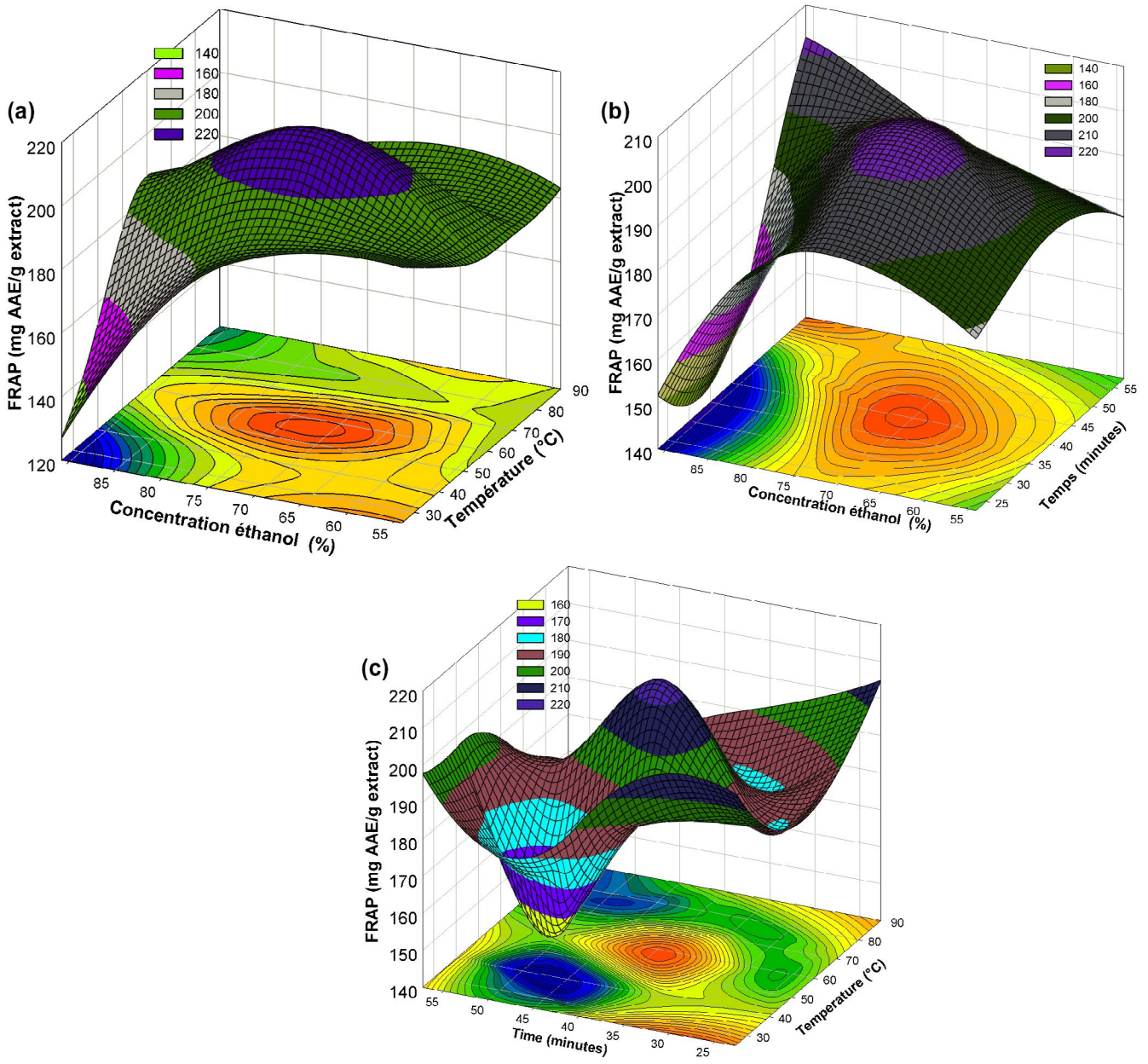

FIGURE 3 Effects of extraction temperature, time, and ethanol concentration on the Ferric reducing antioxidant power (FRAP) of roasted *M. oleifera* leaf extracts

respected simultaneously in *M. oleifera* roasted leaf extract. Hence, the 3D contours were plotted for every dependent variable as a function of extraction temperature and ethanol concentration, while maintaining extraction time for 40 min which is the optimal range for most of the variables studied. The contour plots were superimposed into a single graph and the optimal conditions maximizing each of the four dependent variables identified. The area where all dependent variables were simultaneously optimal was selected and hatched (Figure 5).

In this overall optimum area, the desirability was 0.8–0.96, evidence of how close the fitted optimal area is to the desired one given that the higher the desirability, the better the fitness. The optimal conditions obtained from this procedure are temperature range from 55 to 65°C, ethanol concentration of 65%–75% and extraction time of 40 min.

3.5 | Verification of models in the optimal area

The experimental values for antioxidant activity and TPC obtained in optimal conditions of extraction (60°C, 40 min and 70% ethanol) were compared to the fitted values predicted by the models (Table 4). The closeness of these values provided practical evidence that the reported models explained adequately the influence of studied parameters on antioxidant status of roasted *M. oleifera* leaf extracts. The coefficient of variation was close to zero for every dependent

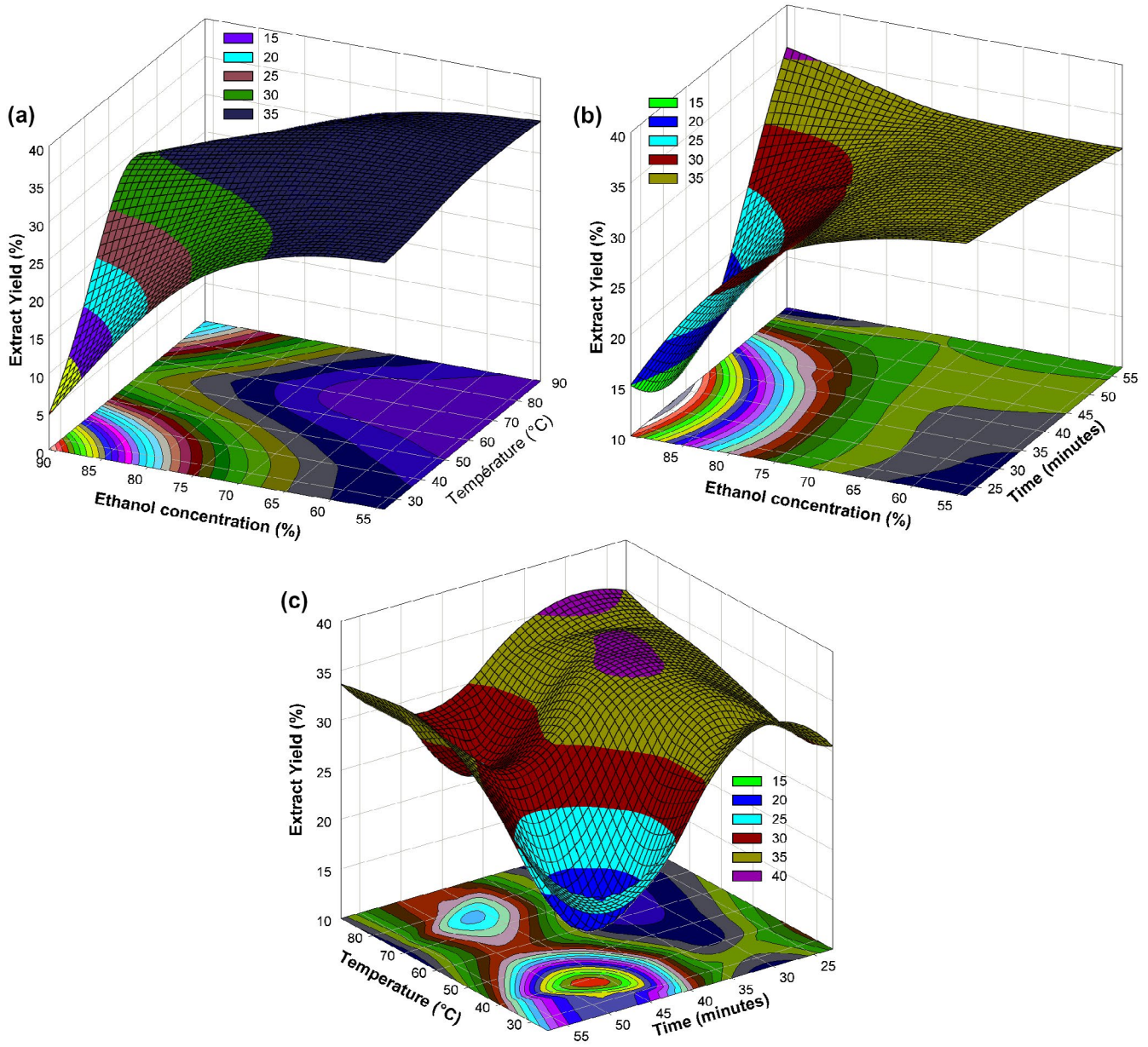

FIGURE 4 Effects of extraction temperature, time, and ethanol concentration on the extraction yield in roasted *M. oleifera* leaf extract

variable indicating that the experimental results were close to the predicted ones. Hence, the second-order polynomial models generated in this study through RSM had good predictive capacity in the studied range of process parameters.

3.6 | Identification and quantification of phenolic compounds and flavonoids in the extracts of roasted *M. oleifera* leaves

HPLC profile of roasted *M. oleifera* leaf extract obtained under optimal conditions showed several peaks among which five phenolic compounds (caffeic acid, rutin, isoquercetin, quercetin, and vanillin) were successfully identified and quantified. Rutin (RT 27.7 min) had the highest concentration (45 mg/g extract) followed by quercetin (15.2 mg/g, RT 38.1 min), isoquercetin (10.4 mg/g, RT 27.1 min.), caffeic acid (9.0 mg/g, RT 20.0 min), and vanillin (4.7 mg/g, RT 3.1 min) (Table 5).

The identified compounds can be divided into two classes: phenolic acids including caffeic acid and vanillin; flavonoids including rutin, isoquercetin, and quercetin. The isoquercetin content in Moringa roasted leaf extract (10.4 mg/g) was higher than 3 mg/g extract obtained in 70% ethanol extract of dried Moringa leaves (Vongsak, Sithisarn, & Gritsanapan, 2013) and 3–8 μg/g found in 70% methanol extracts of germplasm leaves of *M. oleifera* (Nouman et al., 2016). This observation supports the fact that roasting improves phenolic composition of *M. oleifera* leaves. Other phenolic acids such as chlorogenic acid, feruloylquinic acid, gallic acid, and flavonoids such as kaempferol, multiflorin B, catechin, and apigenin identified in Moringa leaf extracts by other authors (Karthivashan,

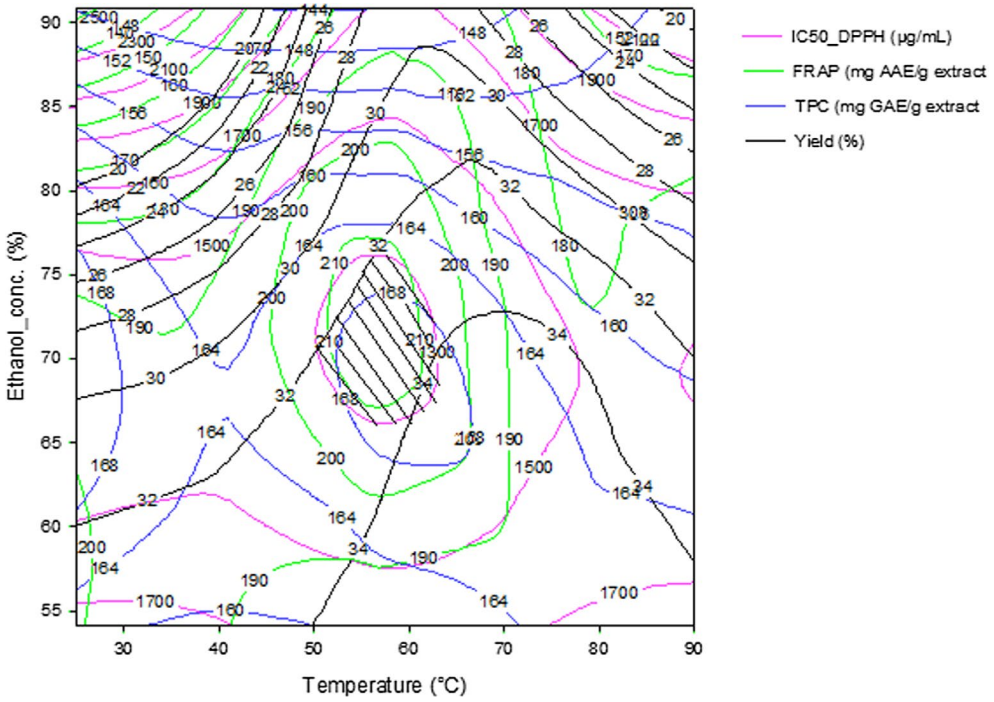

FIGURE 5 Optimal conditions for extraction of antioxidants from roasted *Moringa oleifera* leaves using multiple responses

TABLE 4 Comparison between measured and predicted values in optimal area

	DPPH IC50 (mg/ml)	FRAP (mg AAE/g)	TPC (mg GAE/g)	YIELD (%)
Measured	1.28 ± 0.05	236 ± 10	176 ± 5	33.8 ± 1.0
Predicted	1.25	213	170	33.7
CV	0.02	0.07	0.02	0.002

Abbreviations: CV, coefficient of variation; FRAP, ferric reducing antioxidant power; TPC, total phenolic content.

TABLE 5 Identified phenolics in extract from roasted *M. oleifera* leaves

Retention time[a] (min.)	Maximum UV absorption of sample (nm)	Maximum UV absorption of standard (nm)	Identity	Concentration (mg/g extract)
3.1 ± 0.1	221, 235	219, 235	Vanillin	4.7 ± 0.9
20.0 ± 0.2	219, 247, 321	220, 245, 324	Caffeic acid	9.0 ± 2.0
27.1 ± 0.1	227, 256, 355	221, 255, 356	Isoquercetin	10.4 ± 0.9
27.7 ± 0.2	221, 255, 356	231, 256, 356	Rutin	45.0 ± 5.0
38.1 ± 0.4	214, 256, 369	219, 255, 372	Quercetin	15.2 ± 1.5

[a]Mean value of 5 runs.

Fard, Arulselvan, Abas, & Fakurazi, 2013; Verma, Vijayakumar, Chandra, & Chandana, 2009; Vongsak, Sithisarn, Mangmool, et al., 2013), were not detected in the present study probably due to differences in Moringa variety, as well as other factors like processing method (roasting) and agro-ecological origin.

The AOA and biological activity (antihyperglycemic, anti-diabetic, chemoprotective, etc.) of Moringa have been attributed to its phenolic composition. Quercetin was found to improve insulin-stimulated glucose uptake in mature adipocytes (Figueirinha, Paranhos, Pérez-Alonso, Santos-Buelga, & Batista, 2008), while isoquercetin displays several chemoprotective effects against oxidative stress, cancer, cardiovascular disorders, and diabetes. For instance, isoquercetin was reported to inhibit carbohydrate metabolism enzymes in vitro, such as intestinal α-glucosidase and α-amylase as well as sugar transport mediated by the glucose transporter GLUT2 (Valentová, Vrba, Bancírová, Ulrichová, & Kren, 2014). Rutin, also known as quercetin-3-rutinoside or sophorin, has significant scavenging properties on oxidizing species such as OH radical, superoxide radical, and peroxyl radical (Shrestha, Asgar, Javed, Jasjeet, & Sanjula, 2013). Rutin can lower blood sugar level and blood pressure in diabetic patients (Shrestha et al., 2013). The suggested mechanisms involve either enhancement of

insulin release and sensitivity, or decrease in the expression of resistin which is a hormone linked with obesity and insulin resistance (Shrestha et al., 2013); increase in the expression of peroxisome proliferator-activated receptor (Ahmed, Moneim, & Yazid, 2010) or an enhancement of peripheral glucose utilization by skeletal muscle (Jadhav & Puchchakayala, 2012). These may explain in part the antihyperglycemic (Fombang & Saa, 2016a) and hypotensive (Fombang et al., 2016) effects of *M. oleifera* leaves.

4 | CONCLUSION

The present study has shown that multiple response optimization using RSM is effective for achieving optimal conditions for the extraction of bioactive compounds and antioxidants in roasted leaves of *M. oleifera*. Antioxidant activity was determined using the FRAP and DPPH scavenging activity. Optimal extraction conditions for maximum AOA, TPC and extract yield were 65%–75% ethanol concentration at 55–65°C, for 40 min. Ethanol concentration had the most significant influence on these parameters. HPLC profile of extracts from roasted *M. oleifera* leaves show high amounts of rutin followed by quercetin and isoquercetin, respectively. The antioxidant potential of this extract makes it a potential ingredient for the formulation of nutraceuticals.

ACKNOWLEDGMENTS

The authors gratefully acknowledge The World Academy of Science (TWAS) and the Council for Scientific and Industrial Research (CSIR) for the TWAS-CSIR postgraduate fellowship program (FR number: 3240280447 to Mr. Nobossé). The authors are also thankful to the Director, CSIR-Institute of Himalayan Bioresource Technology, Palampur, H.P. India for providing the research facilities. The financial support of the CSIR, India under projects MLP-0039 and MLP- 0066 is also gratefully acknowledged.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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How to cite this article: Fombang EN, Nobossé P, Mbofung CMF, Singh D. Optimising extraction of antioxidants from roasted *Moringa oleifera* Lam. leaves using response surface methodology. *J Food Process Preserv*. 2020;00:e14482. https://doi.org/10.1111/jfpp.14482