Table 4. Analysis of variance for total flavonoids content.

Source	Sum of squares	Gl	Square medium	F-ratio	P-value
A	41.9539	1	41.9539	41.62	0.0003*
B	6.556	1	6.556	6.5	0.0381*
C	8.09071	1	8.09071	8.03	0.0253*
AA	26.7241	1	26.7241	26.51	0.0013*
AB	2.84387	1	2.84387	2.82	0.1369
AC	0.363804	1	0.363804	0.36	0.5669
BB	1.16451	1	1.16451	1.16	0.3181
BC	0.189728	1	0.189728	0.19	0.6774
CC	3.22117	1	3.22117	3.2	0.117
Total error	7.05561	7	1.00794		
Total (corr.)	94.1556	16			
R-square = 92.50%					
R-square (adjusted by g.l.) = 82.87%					
Standard error of est. = 1.00					
Average absolute error = 0.55					
Durbin–Watson statistic = 2.07 (P = 0.53)					
Lag 1 residual autocorrelation = −0.04					

A = % ethanol; B = temperature (° C); C = time (min).

Table 5. Correlation between dependent variables in *M. oleifera* extracts.

Variable	Phenols	Flavonoids	DPPH$^{\bullet}$	ABTS$^{\bullet+}$
Phenols	1			
Flavonoids	−0.71	1		
DPPH$^{\bullet}$	0.24	0.03	1	
ABTS$^{\bullet+}$	0.62	−0.29	0.70	1

Knowledge of the most effective extraction conditions allows the recovery of bioactive compounds, such as flavonoids [20]. In the present investigation, the extraction by UAE of flavonoid compounds from *M. oleifera* leaves was optimized, 17.52 mg QE/g being obtained by using 90.4% ethanol for 3.2 min at 58.5°C.

These results are comparable with those from previous studies of optimization by UAE in *M. oleifera* leaves that reported concentrations of 25.2 mg QE/g of flavonoids, using 50% ethanol for 5 min [14], and of 14.16 mg QE/g of flavonoids, using 74.5% methanol, for 15 min at 11°C [21].

The extraction of flavonoid compounds was favored at higher concentrations of ethanol, 90.2%. Regarding the temperature, the extraction of both phenolic and flavonoid compounds was maximized at 58.5°C. On the other hand, we observe that the extraction time for flavonoid compounds was less than 3.2 min compared to

36.8 min for phenolic compounds. The times reported by other authors [14,21] to maximize the extraction of flavonoid compounds were 5 and 15 min, respectively; these values are lower than those reported to maximize the extraction of phenolic compounds of 42 and 60 min in other studies [13,18].

It has been pointed out that the longer the extraction time, the lower the extraction yield of some phenolic compounds, which may be due to oxidation that occurs when ultrasonic irradiation is used [22]. The flavonoid compounds obtained in the present study could present oxidation effects at a longer extraction time, so it is important to control this parameter at 3.2 min as indicated by the optimization. Likewise, it is important to use adequate temperatures since temperatures higher than 58.5°C could promote the degradation of the soluble substances extracted and therefore the yield of phenolic compounds [23].

According to the results of the optimization of individual response variables, the process conditions to maximize the yields of the phenolic and flavonoid compounds are different with respect to ethanol concentration and time. An analysis was carried out to determine the correlation of phenols and flavonoids with the antioxidant activity by capture of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals. The correlation coefficients (R) are shown in Table 5. A strong positive correlation of 0.62 can be observed between phenols and inhibition of $ABTS^{\bullet+}$ radical. Although a weak correlation of 0.24 was obtained between phenols and inhibition of the $DPPH^{\bullet}$ radical, a high correlation of 0.70 was observed between the $ABTS^{\bullet+}$ radical and $DPPH^{\bullet}$, which indicated that the extraction of phenols favors the antioxidant activity of the extracts. A high correlation of phenols with the antioxidant activity of extracts from leaves of *M. oleifera* and other plants was also documented [24]. There was no correlation between the flavonoid compounds and the antioxidant activity by capturing the $DPPH^{\bullet}$ radical, since a value of 0.03 was obtained, and there was even a negative correlation between the flavonoids and the $ABTS^{\bullet+}$ radical, which was −0.29; this indicated that the greater the extraction of flavonoid compounds, the lower the antioxidant activity.

The correlation between phenols and flavonoids was −0.71; although this is a strong correlation, it is negative, which indicates that when the extraction of phenolic compounds increases, the extraction of flavonoids decreases and vice versa. The decrease in antioxidant activity when a greater number of flavonoids is extracted is due to the flavonoids in *M. oleifera* leaf extracts being found in their glycosidic forms [13], which possibly interferes with the antioxidant activity *in vitro*. However, when ingested these compounds present different structural transformations, due to acid hydrolysis in the stomach or due to different enzymes from microbiota present in the large intestine, resulting in smaller and biologically active molecules due to the deglycosylation of flavonoids and release in aglycones, which provide flavonoids their function as an antioxidant molecule among other cellular functions [25].

Flavonoids are molecules of great medical relevance due to their different beneficial functions in the body; however, it is also important that phenolic extracts have good antioxidant activity *in vitro*, since this is highly valued in the food industry, because phenolic extracts act as functional ingredients, fulfilling a double function, as an antioxidant in food and as an antioxidant in the body.

Therefore, to enhance the extraction of both flavonoid and phenolic compounds, all the responses were simultaneously optimized, to obtain a more balanced extract and thus enhance its effects.

Simultaneous optimization: The desirability function was used, which allowed determination of the optimal conditions for all the responses studied simultaneously [26]. The scale of the desirability function ranges from 0, a completely undesirable answer, to 1, a totally desired response [27]. A desirability value of 0.82 was obtained,

the response variables were simultaneously optimized, and optimal process conditions of 52.2% ethanol, 3.2 min and 58.7°C were obtained; these effects are shown in the three-dimensional response surface diagram (Figure 3), which allowed simultaneous maximization of all responses, and the model predicted values of 14.55 mg GAE/g for phenols, 10.15 mg QE/g for flavonoids, and 75.69% antiradical activity against $ABTS^{\bullet+}$ and 57% antioxidant activity against $DPPH^{\bullet}$ (Table 6).

The adequacy of the prediction model was evaluated by performing experiments in triplicate under optimized conditions and comparing the predicted values with the experimental ones. Table 6 shows that the observed and predicted values were consistent. The strong correlation between these results confirms the suitability of the model to reflect the intended simultaneous optimization, since the disparity between experimental and model-predicted values remains within a margin of close to 5% for all responses. Suggesting that the CCD-RSM methodology can be effectively used to optimize *M. oleifera* leaves extraction parameters.

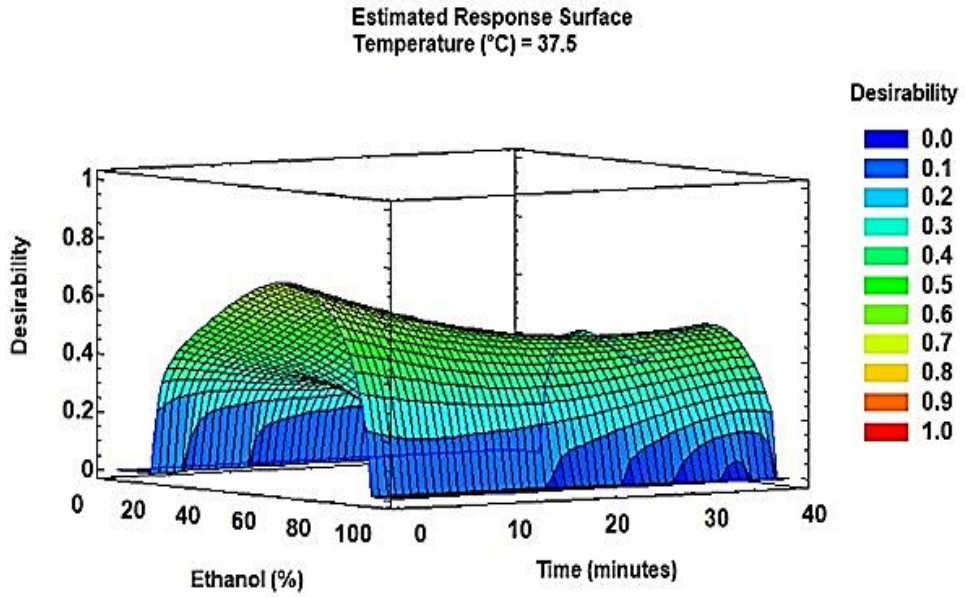

Figure 3. Response surface diagram for simultaneous optimization.

Table 6. Simultaneous optimization in *M. oleifera* extracts.

Response variable	Predicted value by simultaneous optimization1	Experimental value by simultaneous optimization[1,2]
Phenols (mg GAE/g)	14.55	13.92±0.21
Flavonoids (mg QE/g)	10.15	10.60±0.06
$ABTS^{\bullet+}$ (mg GAE/g)	7.95	7.71±0.17
% Inhibition (10 mg)	75.69	72.81±1.58
$DPPH^{\bullet}$ (mg GAE/g)	5.30	4.90±0.19
% inhibition (10 mg)	57.00	52.22±2.01

[1]52.4% ethanol; 58.5°C; 3.2 min. Desirability 0.82. [2]Data for phenols, flavonoids, $DPPH^{\bullet}$, and $ABTS^{\bullet+}$ are the average of triplicates.

The extracts obtained from the *M. oleifera* leaves in this work presented an important source of phenolic compounds; in addition, due to the simultaneous optimization, it was possible to obtain an extract with high antioxidant activity. This is the first study that simultaneously evaluates the effect of process variables

% ethanol, time, and temperature on the UAE of phenols and flavonoids, and in turn, how they influence and correlate with its antioxidant activity.

These types of studies provide important data for the use of *M. oleifera* leaves and in the future could contribute to generating value chains that allow the development of new products in the food sector, such as functional foods. These foods have currently acquired greater importance because their consumption is associated with "promoting optimal health and reducing the risk of chronic/viral diseases and controlling their symptoms"[28].

CONCLUSIONS

The use of an ultrasound-assisted process enhanced the extraction of the phenolic compounds. It was shown that the process variable that had the greatest influence on the extraction of phenolic compounds was the ethanol concentration, followed by the time, and finally the temperature, to maximize the extraction of antioxidant phenolic compounds from *M. oleifera* leaves. The findings presented in this work can contribute to the development of foods and/or nutraceuticals with high phenolic and antioxidant content, to counteract the increase in chronic-degenerative diseases and help maintain a good state of health in the population.

Abbreviations: $ABTS^{\bullet+}$:2,2'-azino-bis(3-ethylbenzothiazoline 6-sulfonic acid), CCD-RMS: Composite Central Design of Response Surface Methodology, $DPPH^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical, mg GAE/g: milligrams of gallic acid equivalents per gram of dry sample, mg QE/g: milligrams of quercetin equivalents per gram of dry sample, *M. oleifera: Moringa oleifera,* UAE: ultrasound-assisted extraction.

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Author contributions: ETR and HEMF carried out the research and wrote the manuscript.

REFERENCES

1. Bhattacharya A, Tiwari P, Sahu PK, Kumar S: A review of the phytochemical and pharmacological characteristics of *Moringa oleifera*. J. Pharm. Bioallied Sci. 2018, *10*(4):181. DOI: https://doi.org/10.4103/JPBS.JPBS_126_18
2. Castro-López C, Ventura-Sobrevilla JM, González-Hernández MD, Rojas R, Ascacio-Valdés JA, Aguilar CN, Martínez-Ávila GC: Impact of extraction techniques on antioxidant capacities and phytochemical composition of polyphenol-rich extracts. Food Chem 2017, 237:1139–48. DOI: https://doi.org/10.1016/j.foodchem.2017.06.032
3. Alkhudhayri DA, Osman MA, Alshammari GM, Al Maiman SA, Yahya MA: *Moringa peregrina* leaf extracts produce anti-obesity, hypoglycemic, anti-hyperlipidemic, and hepatoprotective effects on high-fat diet fed rats. Saudi J Biol Sci 2021, 28(6):3333-3342. DOI: https://doi.org/10.1016/j.sjbs.2021.02.078
4. Mabrouki L, Rjeibi I, Taleb J, Zourgui L: Cardiac ameliorative effect of *Moringa oleifera* leaf extract in high-fat diet-induced obesity in rat model. BioMed Res Intern 2020, e6583603. DOI: https://doi.org/10.1155/2020/6583603
5. Jalili F, Jafari SM, Emam-Djomeh Z, Malekjani N, Farzaneh V: Optimization of ultrasound-assisted extraction of oil from canola seeds with the use of response surface methodology. Food Anal Methods 2018, 11:598–612. DOI: https://doi.org/10.1007/s12161-017-1030-z
6. Zhong J, Wang Y, Yang R, Liu X, Yang Q, Qin X: The application of ultrasound and microwave to increase oil extraction from *Moringa oleifera* seeds. Industrial Crops Products, 2018, 120:1-10. DOI: https://doi.org/10.1016/j.indcrop.2018.04.028

7. Hossain MB, Brunton NP, Patras A, Tiwari B, O'Donnell CP, Martin-Diana AB, Barry-Ryan C: Optimization of ultrasound assisted extraction of antioxidant compounds from marjoram (*Origanum majorana* L.) using response surface methodology. Ultrason Sonochem 2012, 19:582–590. DOI: https://doi.org/10.1016/j.ultsonch.2011.11.001
8. Altemimi A, Watson DG, Choudhary R, Dasari MR, Lightfoot DA: Ultrasound assisted extraction of phenolic compounds from peaches and pumpkins. *PloS one* 2016, 11(2), e0148758. DOI: https://doi.org/10.1371/journal.pone.0148758
9. Makkar HPS, Blümmel M, Borowy NK, Becker K: Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J Sci Food Agric 1993, 61:161–5. DOI: https://doi.org/10.1002/jsfa.2740610205
10. Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M, Cazin M, Cazin J-C, Bailleul F, Trotin F: Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. J. Ethnopharm. 2000, 72(1–2):35–42. DOI: https://doi.org/10.1016/s0378-8741(00)00196-3
11. Randhir R, Shetty K: Mung beans processed by solid-state bioconversion improves phenolic content and functionality relevant for diabetes and ulcer management. Innov Food Sci Emerg Technol 2007, 8:197–204. DOI: https://doi.org/10.1016/j.ifset.2006.10.003
12. Re R, Pellegrini N, Proteggente N, Pannala A, Yang M, Rice-Evans R: Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 1999, 26:1231–7. DOI: https://doi.org/10.1016/s0891-5849(98)00315-3
13. Pollini L, Tringaniello C, Ianni F, Blasi F, Manes J, Cossignani L: Impact of ultrasound extraction parameters on the antioxidant properties of *Moringa oleifera* leaves. Antioxidants, 2020, 9(4):277. DOI: https://doi.org/10.3390/antiox9040277
14. Pereira JM, Viell FLG, de Lima PC, Silva E, Pilau EJ. Corrêa RC, Bona E, Vieira AM: Optimization of the extraction of antioxidants from Moringa leaves: A comparative study between ultrasound-and ultra-homogenizer-assisted extractions. J Food Process Preserv 2021, 45(6), e15512. DOI: https://doi.org/10.1111/jfpp.15512
15. Gómez-Martínez M, Ascacio-Valdés JA, Flores-Gallegos AC, González-Domínguez J, Gómez-Martínez S, Aguilar CN, Morlett-Chávez JA, Rodríguez-Herrera R: Location and tissue effects on phytochemical composition and in vitro antioxidant activity of *Moringa oleifera*. Industrial Crops and Products 2020, 151: 112439. DOI: https://doi.org/10.1016/j.indcrop.2020.112439
16. Barba FJ, Putnik P, Kovačević DB, Poojary MM, Roohinejad S, Lorenzo JM, Koubaa M: Impact of conventional and non-conventional processing on prickly pear (*Opuntia* spp.) and their derived products: From preservation of beverages to valorization of byproducts. Trends Food Sci Technol 2017, 67:260–70. DOI: https://doi.org/10.1016/j.tifs.2017.07.012
17. Chakraborty S, Uppaluri R, Das C. Optimization of ultrasound-assisted extraction (UAE) process for the recovery of bioactive compounds from bitter gourd using response surface methodology (RSM). Food Bioprod Process 2020, 120:114–22. DOI: https://doi.org/10.1016/j.fbp.2020.01.003
18. Zhao B, Deng J, Li H, He Y, Lan T, Wu D, Gong H, Zhang Y, Chen Z: Optimization of phenolic compound extraction from Chinese *Moringa oleifera* leaves and antioxidant activities. J Food Qual 2019, e5346279. DOI: https://doi.org/10.1155/2019/5346279
19. Rocchetti G, Blasi F, Montesano D, Ghisoni S, Marcotullio MC. Sabatini S, Cossignani L, Lucini L: Impact of conventional/non-conventional extraction methods on the untargeted phenolic profile of *Moringa oleifera* leaves. Food Res Int 2019, 115:319–27. DOI: https://doi.org/10.1016/j.foodres.2018.11.046
20. Wang T, Li Q, Bi K: Bioactive flavonols in medical plants: structure, activity and biological fate. Asian J Pharm Sci 2018, 13:12–23. DOI: https://doi.org/10.1016/j.ajps.2017.08.004
21. Yerena-Prieto BJ, Gonzalez-Gonzalez M, Vázquez-Espinosa M, González-de-Peredo AV, García-Alvarado MA, Palma M, Rodríguez-Jimenes GdC, Barbero GF: Optimization of an ultrasound-assisted extraction method applied to the extraction of flavonoids from Moringa leaves (*Moringa oleífera* Lam.). Agronomy, 2022, 12(2):261. DOI: https://doi.org/10.3390/agronomy12020261
22. Daghaghele S, Kiasat AR, Safieddin Ardebili SM, Mirzajani R: Intensification of extraction of antioxidant compounds from *Moringa oleifera* leaves using ultrasound-assisted approach: BBD-RSM design. Int J Fruit Sci 2021, 21(1):693–705. DOI: https://doi.org/10.1080/15538362.2021.1926396
23. Wang W, Yang J, Yang J: Optimization of ultrasound-assisted aqueous two phase extraction of polyphenols from olive leaves. Preparative Biochem Biotechnol 2021, 51(8):821–31. DOI: https://doi.org/10.1080/10826068.2020.1861012
24. Mwamatope B, Tembo D, Chikowe I, Kampira E, Nyirenda C: Total phenolic contents and antioxidant activity of *Senna*

singueana, *Melia azedarach*, *Moringa oleifera* and *Lannea discolor* herbal plants. Sci African 2020, 9, e00481. DOI: https://doi.org/10.1016/j.sciaf.2020.e00481

25. Żyżyńska-Granica B, Gierlikowska B, Parzonko A, Kiss AK, Granica S: The bioactivity of flavonoid glucuronides and free aglycones in the context of their absorption, II phase metabolism and deconjugation at the inflammation site. Food Chem Toxicol 2020, 135, e110929. DOI: https://doi.org/10.1016/j.fct.2019.110929
26. Zainal WNHW, Azian NAAM, Albar SS, Rusli AS: Effects of extraction method, solvent and time on the bioactive compounds and antioxidant activity of *Tetrigona apicalis* Malaysian propolis. J Apic Res 2022, 61(2):264–70. DOI: https://doi.org/10.1080/00218839.2021.1930958
27. Candioti LV, De Zan MM, Cámara MS, Goicoechea HC: Experimental design and multiple response optimization. Using the desirability function in analytical methods development. Talanta, 2014, 124:123–38. DOI: https://doi.org/10.1016/j.talanta.2014.01.034
28. Martirosyan D., Kanya H., Nadalet C. Can functional foods reduce the risk of disease? Advancement of functional food definition and steps to create functional food products. Functional Foods in Health and Disease 2021; 11(5): 213-221. DOI: https://www.doi.org/10.31989/ffhd.v11i5.788