ORIGINAL RESEARCH PAPER

OPTIMIZATION OF THE MICROWAVE ASSISTED EXTRACTION AND BIOLOGICAL ACTIVITIES OF POLYPHENOLS FROM LEMON VERBENA LEAVES

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Abstract

The present study aims to optimize the extraction of phenolics by microwave-assisted extraction (MAE) using the response surface methodology (RSM), from *Lemon verbena* leaves. The optimized extract was tested for its antioxidant activity using two methods (DPPH and reducing power) and its antibacterial efficiency by using disk diffusion assay and broth microdilution, against two Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and two Gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633) strains. Under the optimized conditions (40% (v/v) of ethanol concentration, 188 s of irradiation time, 600 W of microwave power and 1:40 g/mL of solid-to-liquid ratio) the total phenolic content (TPC) was 67.87 ± 1.61 mg GAE/g DW. The IC_{50} of the extract was 139.65 ± 1.44 µg/mL and 56.60 ± 2.79 µg/mL for DPPH inhibition and reducing power, respectively. The best antibacterial activity was shown by the extract obtained by MAE with lower MBC (1.56 to 18.75 mg/mL) and MBC/MIC ratio. *Lemon verbena* extract can be used as an ingredient in cosmetics, food supplements and herbal medicinal products due to its interesting biological properties.

Keywords: lemon verbena leaves, phenolic compounds, optimization, microwave-assisted extraction, antioxidant activity, antibacterial activity

Introduction

The *Verbenaceae* family is one of the most important families in the plant kingdom. It has several economic values due to its numerous uses in the food

industries (Pérez Zamora *et al.,* 2018; Moshari-Nasirkandi *et al.,* 2020). Indeed, species of this family are used as food supplements (Mari *et al.,* 2012), sweetener (Combrinck *et al.,* 2007), food flavoring (De Almeida *et al.,* 2018), and edible coating for food storage and preservation (Ishkeh *et al.,* 2019; Castro *et al.,* 2011; Montanari *et al.,* 2011), spice (Sarrazin *et al.,* 2015), they are also used in food seasoning (Marongiu *et al.,* 2010). In Addition, since ancient times, the plants from this family are known as aromatic species for ornamental use or in folk medicine. *Lemon verbena* is one of the verbenaceae plants which has several therapeutic virtues, biological activities (Mashayekhi-sardoo *et al.,* 2020; Bekara *et al.,* 2020) and sedative effect that acts especially on the central nervous system (Bekara *et al.,* 2020). It has a wide geographical distribution from South America to North Africa and South of Europe (Mashayekhi-sardoo *et al.,* 2020), and its uses date back to the Inca civilization (Elechosa *et al.,* 2017). It is usually consumed as an infusion for its stimulating, relaxing properties and exalting smell (Rocha *et al.,* 2019). Its leaves have a pleasant lemon smell when crushed, and they are used to flavor fish, poultry, salads, jams, puddings, and soft drinks (Funes *et al.,* 2010; Cunha *et al.,* 2012). Its aromas are also used in perfumes and potpourri to scent homes (Brant *et al.,* 2010). Due to the pleasant lemony fragrance and its application in food industries and cosmetics, as well as its use as a home remedy for several health problems, the plant is currently available in other parts of the world, as well (Bahramsoltani *et al.,* 2018). Several categories of phytochemicals have been identified in its different parts such as terpenes, phenylethanoids and phenylpropanoids, flavonoids, miscellaneous compounds (Bahramsoltani *et al.,* 2018) and phenolics (Pereira *et al.,* 2017; Bekara *et al.*, 2020). These latter are one of the most significant compounds which confer bioactive potential to plants (Moshari-Nasirkandi *et al.,* 2020). For this reason, recent research has focused on improving the methods of their extraction, in order to optimize their recovery quantitatively and qualitatively (Leyva-Jiménez *et al.,* 2020b).

Several classical extraction processes have been used for the extraction of polyphenols from *Lemon verbena* leaves (Cheurfa and Allem, 2016; Jalal *et al.,* 2019; Leyva-Jiménez *et al.,* 2020b). MAE as a green technology is one of the most efficient eco-extraction methods, because it saves time, energy and solvent (Ekezie *et al.,* 2017). MAE allows high extraction efficiency of TPC. This can generally be attributed to its heating effect, which occurs due to the dipole rotation of the solvent in the microwave field. This causes the solvent temperature to rise, which then increases the solubility of the compound of interest (Hayat *et al.,* 2009). This process is used for the extraction of phytochemicals for the pharmaceutical and food industries (Dahmoune *et al.,* 2015; Hayat *et al.,* 2009). The RSM is a collection of statistical techniques used in many engineering applications to improve and to optimize processes. It is effective for responses that are influenced by several factors and their interactions (Liu *et al.,* 2015). Several recent works had studied the optimization of response (s) from *Lemon verbena* leaves using RSM (Ivanović *et al.,* 2018; Leyva-Jiménez *et al.,*2020a, Leyva-Jiménez *et al.,* 2020b; Villegas-Aguilar *et al.,* 2020; Valiyan *et al.,* 2021).

Recently, many serious side impacts in humans were reported by synthetic preservatives such as cancer and cardiovascular disorders (Mohammadzadeh-Aghdash *et al.,* 2019*;* Atta *et al.,* 2017). So, an increased attention was focused on the potential use of natural products, such as extracts of medicinal plants, in food industry. Therefore, medical herb extracts have the potential of being used in this field. The objective of this work was to optimize the extraction of phenolic compounds by MAE, using green extraction method, from *Lemon verbena* leaves, and to determine the total flavonoid content (TFC), the antioxidant activity and the antibacterial capacity of the optimized extract with potential bioactive interest. The results were then compared to those obtained by conventional extraction method.

Materials and methods

Reagents and plant material

The chemicals were obtained from Biochem Chemopharma and Sigma Aldrich. The *Lemon verbena* leaves were collected from July to August (2018), in the region of Toudja (Bejaia, Algeria). They were washed, dried at 40 °C, until the stabilization of the weight, and then grounded. The powder was passed through 125, 250 and 500 μm sieves and stored in the dark at 4 °C.

Phytochemical analyses

Microwave-assisted extraction (MAE)

The extraction of polyphenols was carried out using a laboratory microwave with a modified system (2450 MHz, Maxipower Model MAXMO23S, China), having a maximum power of 900 W. One gram of powder was mixed with the solvents (water, acetone, ethanol, and methanol) at different concentrations (from 20 to 80 % v/v) and solid-to-solvent ratios (from 15 to 45 g/mL). The mixtures, exposed to a different microwave powers ranging from 200 to 700 W for extraction, were filtered and stored at 4°C. The parameters with the highest influence on the TPC microwave extraction were selected for the RSM study.

Maceration extraction (ME)

This extraction was achieved by adopting the protocol of Cheurfa and Allem. (2016). One gram of powder was mixed with 40 mL of 40% ethanol (v/v), the mixture was shaken well and left to macerate for 3 days at room temperature, then the filtrate was stored at 4°C.

Determination of total phenolic content

The determination of the TPC was carried out using the Folin-Ciocalteu method as described by Georgé *et al*. (2005). The TPC was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW).

Determination of total flavonoid content

The total flavonoid content (TFC) was determined by the method of Quettier-Deleu *et al*. (2000). The content was expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g DW).

Experimental design

The response surface methodology is a statistical approach that was carried out to define the effects of different variables on the microwave extraction process, in order to optimize the TPC extraction. A central composite design (CCD) was developed using the RSM, to optimize the TPC extraction yield. The factors corresponding to each independent variable were: X_1-solvent concentration (%, v/v), X_2-irradiation time (s), X_3-microwave power (W) and X_4-solid-to-solvent ratio (mg/mL). The TPC yield was the dependent response variable (Y). A second-order polynomial model was realized to predict the optimal conditions for extracting polyphenols (Equation 1):

$$Y = ß_{\circ} + \sum_{i=1}^{k} ß_i X_j + \sum_{i=1}^{k} ß_{ii} X^2 \sum_{i>j}^{k} ß_{ij} X_i X_j + E \qquad (1)$$

where $ß_{\circ}$ is the constant coefficient of the model, $ß_i$, $ß_{ii}$ and $ß_{ij}$ are the coefficients of linear, quadratic and interactive terms, respectively, X_i and X_j represent the coded independent variables.

Biological activities

Antioxidant activity

Two methods were applied to assess the antioxidant activity of the extract: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP) (Brand-Williams *et al*., 1995; Yildirim *et al*., 2001). These activities were expressed in terms of IC_{50} (µg/mL), where the IC_{50} of DPPH scavenging activity is the concentration of sample or standard that inhibit 50% of DPPH radicals, it was obtained by linear regression analysis of dose-response curve plotting between the % of inhibition and concentration. For reducing power, the IC_{50} is the extract concentration where the absorbance is 0.5, and is calculated from the graph of absorbance at 700 nm against the extract concentration (Rezig *et al.,* 2019). The lowest IC_{50} means that the sample had the highest antioxidant capacity (Fidrianny *et al.,* 2015). All the assays were carried out in triplicate; gallic acid was used as the positive control.

Antibacterial activity

Bacterial strains. MAE and ME were tested for their antibacterial activities against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633 strains, provided by the Pasteur Institute (Algeria).

Agar diffusion method. The antibacterial effect of the extracts was tested using the disk diffusion method (Wikler, 2006). Each bacterial suspension with a concentration of 10^6 CFU/mL (at 625 nm) was cultured on plates containing Mueller-Hinton medium. The paper disks (6 mm) were imbibed with 20 µL of extract (100 mg/mL) prepared in the DMSO and placed on the inoculated agar. The plates were incubated at 37 °C for 24 h. DMSO-impregnated discs were used as the negative control. The antibacterial activity was determined by measuring the inhibition zones (IZ) in millimeters (mm), an extract is active when its IZ around

the disk is greater than 6 mm (Amiour *et al*., 2014). The experiments were performed in duplicate.

MIC and MBC. The MIC was determined by the method of Wikler (2006). The dilutions ranging from 25 to 0.05 mg/mL were performed using 96-well microplates device. However, the MBC was determined from the MIC values. The DMSO was used as the negative control. The microplates were incubated at 37 °C for 24 h and the tests were performed in triplicate. The MIC is the lowest concentration of the extract required to completely inhibit the growth of the bacteria and the MBC is the concentration required to kill them.

Statistical analysis

The experiments were performed in triplicate, the influence of each factor on the TPC yield for the MAE, was statistically evaluated by the analysis of variance (ANOVA) and the Tukey's post hoc test with a 95% confidence level. To construct the CCD approach, JMP software (version 10.0, SAS, USA) was used. The Tukey's post hoc test ($p < 0.05$) was also used to compare the antioxidant activity and the antibacterial activity of the extracts obtained with MAE and ME processes.

Results and discussion

Effect of independent variables

Microwave extraction is influenced by several parameters such as: particle size, extraction time, solid-to-solvent ratio, microwave power and type of solvent. The preliminary study results of the microwave-assisted extraction of TPC are shown in Table1.

Effect of particle size

The polyphenols extraction rate increased with the decrease of the particle size. According to Table 1, the particle size that resulted in a maximum extraction efficiency was 125 µm (64.84±1.82 mg GAE/g DW). Indeed, the extraction rate is increased since the diffusion distance of the solute within the solid is decreased when the particle size is smaller (Pinelo *et al*., 2005; Çavdar *et al.,* 2017). 125 µm was chosen to evaluate the effect of the extraction solvent on the TPC yield.

Effect of extraction solvent

The extraction efficiency of polyphenols depends on two processes. First, the solubility of the biomaterials of interest, through the interaction between the solvent and the plant matrix. Second, the microwave energy absorption properties of the solvent, which is determined by its dielectric constant (Dahmoune *et al.,* 2015).

The extraction with methanol and ethanol solvents gave the best TPC yields, with statistically equal values. The ethanol was chosen for the RSM assays, because it is the most widely used solvent due to its low toxicity, and it could be used safely in the food, pharmaceutical and cosmetic industries (Yuan *et al.,* 2019; Guemghar *et al.,* 2020; Neshat *et al.,* 2020).

Table 1. Results of single-factor experiments for the MAE of TPC.

Particle size		Type of solvent		Solvent concentration		Irradiation time		Microwave power		Solid-to-solvent ratio	
μm	TPC yield (mgGAE/gDW)	Type	TPC yield (mgGAE/gDW)	% (v/v)	TPC yield (mgGAE/gDW)	s	TPC yield (mgGAE/gDW)	W	TPC yield (mgGAE/gDW)	g/mL	TPC yield (mgGAE/gDW)
125	64.84±1.82[a]	EtOH	54.00±1.18[ab]	20	48.74±1.14[c]	90	54.40±1.25[c]	200	55.43±1.00[ab]	1:15	54.56±1.46[c]
250	56.00±1.55[b]	MeOH	57.69±1.40[a]	40	50.31±1.02[bc]	120	54.70±1.16[c]	300	57.53±0.55[a]	1:20	57.22±0.77[b]
500	55.33±1.73[b]	Acetone	52.76±1.40[b]	50	51.52±1.64[b]	150	59.13±1.59[b]	400	53.78±1.09[b]	1:25	61.95±0.96[a]
		Water	46.04±1.10[c]	60	55.71±1.26[a]	180	64.37±1.51[a]	500	53.46±0.99[b]	1:30	60.48±1.01[a]
				80	55.53±1.06[a]	210	55.18±1.70[c]	600	51.10±1.21[c]	1:35	62.73±1.14[a]
						240	49.33±1.46[d]	700	50.23±1.15[c]	1:40	57.67±1.05[b]
										1:45	54.41±0.48[c]

Values are mean ± standard deviation. Values with different letters (a, b, c) were significantly different (Tukey, $p < 0.05$).

Effect of ethanol concentration

The presence of water in the solvent facilitates heat distribution throughout the sample, which enhances the dissolution and extraction yield of polyphenols (Guemghar *et al.,* 2020). However, low concentration of ethanol can also induce a low extraction of TPC, probably due to the difference in dielectric properties of the solvent towards microwave heating (Dahmoune *et al.,* 2015; Simić *et al.,* 2016). So, it is necessary to find an appropriate concentration to obtain a better extraction rate. In this study, ethanol 60 and 80% gave the maximum TPC yields, and ethanol concentration of 60% was set for the following single-factor experiments. The range 40-80% was chosen for the CCD tests.

Effect of irradiation time

The irradiation time has a significant effect on the extraction rate of phenolic compounds by the fact that the extraction duration is proportional to the extraction yield (Dahmoune *et al.,* 2015; Djaoud *et al.,* 2020). In this study, the results showed that the extraction yield of TPC increased with the increase in the irradiation time, and reached its maximum after 180 s. Beyond this value, the extraction yield decreased progressively as the irradiation time was prolonged. This decrease is probably due to the thermal degradation of polyphenols (Dahmoune *et al.,* 2014). Thus, 180 s was the optimal point used for the single-factor tests, and the range 150-210s was used to perform the RSM tests.

Effect of microwave power

The microwave power effect on the polyphenols extraction yield was performed between 200 and 700 W. The TPC yield increase from 200 W to reach a maximal level at 300 W, after that, it begins to decrease with further increase of microwave power until 700 W. Microwave power intensity, controls the amount of energy supplied to the sample that is converted into thermal energy. It also affects the interactions and distribution of the analytes between the sample and the solvent (Hayat *et al.,* 2009; Lefsih *et al.,* 2017). The increase of microwave power, increased the extraction efficiency of polyphenols. However, a high microwave power can increase the temperature of the processed product that leads to a thermal degradation of the compounds (Guemghar *et al.,* 2020). Hence, 300 W was selected as the optimal level used for the single-factor tests, and 200-600 W was the range used to perform CCD design.

Effect of solid-to-solvent ratio

The TPC increased as the solid-to-solvent ratio increases progressively up to a maximum of 1:35 g/mL. Subsequently, the extraction efficiency decreased as the ratio increased. Indeed, increase in solid-to-solvent ratio decelerated mass transfer resulting from the lower heating efficiency under microwave conditions and the solubility of polyphenols (Dahmoune *et al.,* 2015). In the same perspective, this can be explained by the fact that a larger volume of solvent requires greater absorption of microwave energy, but this energy may not be sufficient to destroy the cell walls and release the target components. The range 20-40 g/mL was used to realize the CCD design.

Optimization by RSM

An experimental central composite design (CCD) was carried out based on the ethanol concentration, irradiation time, microwave power and solid-to-solvent ratio as the independent variables (X_1, X_2, X_3 and X_4, respectively), the response (Y) represents the TPC. The values of the responses to different experimental combinations of coded variables are shown in Table 2.

Thirty experiments were performed with three levels for each factor, in order to study the influence of each of them and the result of their interactions. The levels of the independent variables were chosen based on the values obtained in the single-factor experiments.

Modeling and model fitting

In this study, the least square technique was used to calculate the regression coefficients of the intercept, linear, quadratic and interaction terms of the model (Table 3) as mentioned by Zhang *et al.* (2013). The *p*-value is used to check the significance of each coefficient, and the interaction pattern between the variables. The linear parameters which are X_1, X_2, X_3, quadratic effects X_2^2 and X_4^2 as well as interactions X_1X_3, X_2X_3 and X_2X_4 were highly significant ($< 0.0001^*$). Solid-to-solvent ratio X_4, quadratic effects X_1^2, X_3^2, interactions X_1X_2, X_1X_4 and X_3X_4 were not significant ($p > 0.05$). The *F*-value (72.36) and the *p*-value ($< 0.0001^*$) show that the model is highly significant (Ji *et al.,* 2018).

Table 2. CCD with the experimental and predicted values for the TPC yield using the MAE.

Run	X_1	X_2	X_3	X_4	Recovery of TPC (mg GAE/g DW)	
					Experimental	**Predicted**
1	80 (+1)	150 (-1)	200 (-1)	40 (+1)	46.0±0.90[kl]	47.00
2	60 (0)	180 (0)	400 (0)	30 (0)	54.2±0.20[cdefghi]	53.21
3	80 (+1)	210 (+1)	600 (+1)	20 (-1)	54.8±1.13[cdefgh]	54.59
4	40 (-1)	210 (+1)	600 (+1)	40 (+1)	62.6±1.31[ab]	63.15
5	80 (+1)	210 (+1)	200 (-1)	20 (-1)	47.0±1.41[jkl]	47.59
6	60 (0)	180 (0)	400 (0)	20 (-1)	64.4±2.44[a]	64.41
7	40 (-1)	150 (-1)	200 (-1)	40 (+1)	57.2±1.73[bcdef]	56.45
8	80 (+1)	210 (+1)	600 (+1)	40 (+1)	58.8±1.92[abcde]	58.66
9	40 (-1)	210 (+1)	600 (+1)	20 (-1)	59.2±2.26[abcd]	59,03
10	40 (-1)	150 (-1)	200 (-1)	20 (-1)	59.8±0.41[abc]	60.78
11	80 (+1)	150 (-1)	200 (-1)	20 (-1)	52.9±3.25[efghij]	51.38
12	80 (+1)	180 (0)	400 (0)	30 (0)	48.2±2.88[ijkl]	49.02
13	60 (0)	210 (+1)	400 (0)	30 (0)	46.2±3.12[kl]	45.92
14	60 (0)	180 (0)	600 (+1)	30 (0)	53.8±1.48[cdefghi]	54.24
15	60 (0)	180 (0)	400 (0)	30 (0)	53.6±2.68[defghi]	53.21
16	80 (+1)	150 (-1)	600 (+1)	20 (-1)	51.6±3.44[fghijk]	52.44
17	60 (0)	180 (0)	400 (0)	30 (0)	53.8±0.50[cdefghi]	53.21
18	80 (+1)	150 (-1)	600 (+1)	40 (+1)	50.8±3.11[ghijk]	49.80
19	80 (+1)	210 (+1)	200 (-1)	40 (+1)	50.3±1.55[hijk]	49.91
20	60 (0)	180 (0)	400 (0)	40 (+1)	63.8±1.70[a]	64.27
21	40 (-1)	180 (0)	400 (0)	30 (0)	56.3±1.11[cdefgh]	55.96
22	60 (0)	180 (0)	400 (0)	30 (0)	53.5±2.83[defghi]	53.21
23	60 (0)	150 (-1)	400 (0)	30 (0)	42.7±2.12[l]	43.46
24	60 (0)	180 (0)	400 (0)	30 (0)	53.0±0.28[efghij]	53.21
25	40 (-1)	150 (-1)	600 (+1)	40 (+1)	54.2±0.11[cdefghi]	54.45
26	60 (0)	180 (0)	200 (-1)	30 (0)	51.7±0.14[fghijk]	51.74
27	40 (-1)	150 (-1)	600 (+1)	20 (-1)	57.6±1.40[bcdef]	57.03
28	60 (0)	180 (0)	400 (0)	30 (0)	52.6±1.37[fghij]	53.21
29	40 (-1)	210 (+1)	200 (-1)	40 (+1)	59.2±0.85[abcd]	59.20
30	40 (-1)	210 (+1)	200 (-1)	20 (-1)	56.8±1.36[bcdefg]	56.83

X_1: Ethanol concentration, X_2: Irradiation time, X_3: Microwave power, X_4: Solid-to-solvent ratio, GAE: gallic acid equivalent and DW: dry weight.

a, b, c, d, e, f, g, h, i, j, k, l. Different letters, per column, indicate significant differences between mean values ($p < 0.05$). Equal letters indicate non-significant differences ($p > 0.05$).

The determination coefficient R^2 and the adjusted determination coefficient R^2_{Adj} were 0.9854 and 0.9720, respectively, they were closely related. It demonstrates the good fit of the model to the experimental results. High R^2 value indicates a high percentage of variability in responses that can be explained by these patterns (Ji *et al.,* 2018; Djaoud *et al.,* 2020).

In fact, the obtained R^2 means that 98.54% of the variations in the sample were due to the independent variables, and 1.46% of total variations could not be explained by this model (Song *et al.,* 2011). Low coefficient of variation (C.V.%) of 1.29% indicates a good model reproducibility (Simić *et al.,* 2016).

Table 3. Analysis of ANOVA for the fitted quadratic polynomial model of the TPC using MAE.

Parameter	Estimated coefficients	Standard error	DF	Sum of squares	F-value	Prob> F
Model	53.207895	0.273901	14	787.66551	72.3626	<0.0001*
Intercept						
B₀	53.207895	0.273901		787.66551	72.3626	<0.0001*
Linear						
X₁	-3.472222	0.207833	1	217.01389	279.1179	<0.0001*
X₂	1.2277778	0.207833	1	27.13389	34.8989	<0.0001*
X₃	1.25	0.207833	1	28.12500	36.1737	<0.0001*
X₄	-0.066667	0.207833	1	0.080000	0.1029	0.7528
Quadratic						
X₁²	-0.715789	0.547802	1	1.32746	1.7074	0.2110
X₂²	-8.515789	0.547802	1	187.88928	241.6585	<0.0001*
X₃²	-0.215789	0.547802	1	0.12065	0.1552	0.6992
X₄²	11.13211	0.547802	1	321.19667	413.1152	<0.0001*
Interaction						
X₁/X₂	0.0375	0.22044	1	0.02250	0.0289	0.8672
X₁/X₃	1.2	0.22044	1	23.04000	29.6335	<0.0001*
X₁/X₄	-0.0125	0.22044	1	0.00250	0.0032	0.9555
X₂/X₃	1.4875	0.22044	1	35.40250	45.5338	<0.0001*
X₂/X₄	1.675	0.22044	1	44.89000	57.7364	<0.0001*
X₃/X₄	0.4375	0.22044	1	3.06250	3.9389	0.0658
Lack of fit			10	10.027485	3.0665	0.1140
Pure error			5	1.635000	3.0665	0.1140
Residual			15	11.66249		
R²					0.98541	
R²*Adjusted*					0.971792	
C.V. %	1.2992					
RMSE*	0.8818					
Corr Total			29	799.32800		

The linear effect of the ethanol concentration was significantly negative. Thus, the increase in the ethanol concentration could decrease the extraction yield. However, the linear effect of the irradiation time and microwave power, were significantly positive, which means that their increase possibly increase the extraction yield. The second-order polynomial equation was determined as follows (Equation 2):

$$Y(TPC) = 53.21 - 3.47X_1 + 1.23X_2 + 1.25X_3 - 8.51X_2^2 + 11.13X_4^2 + 1.2X_1X_3 + 1.49X_2X_3 + 1.67X_2X_4 \qquad (2)$$

Response surface analysis

The effect of the independent variables and their cross-effect on the phenolic performance was observed by a three-dimensional response surface curve, which is shown in Figure 1 A-F. The response was plotted on the z-axis with respect to the two studied independent variables, while keeping the other two remaining independent variables at their zero levels (Hayat *et al.*, 2009).
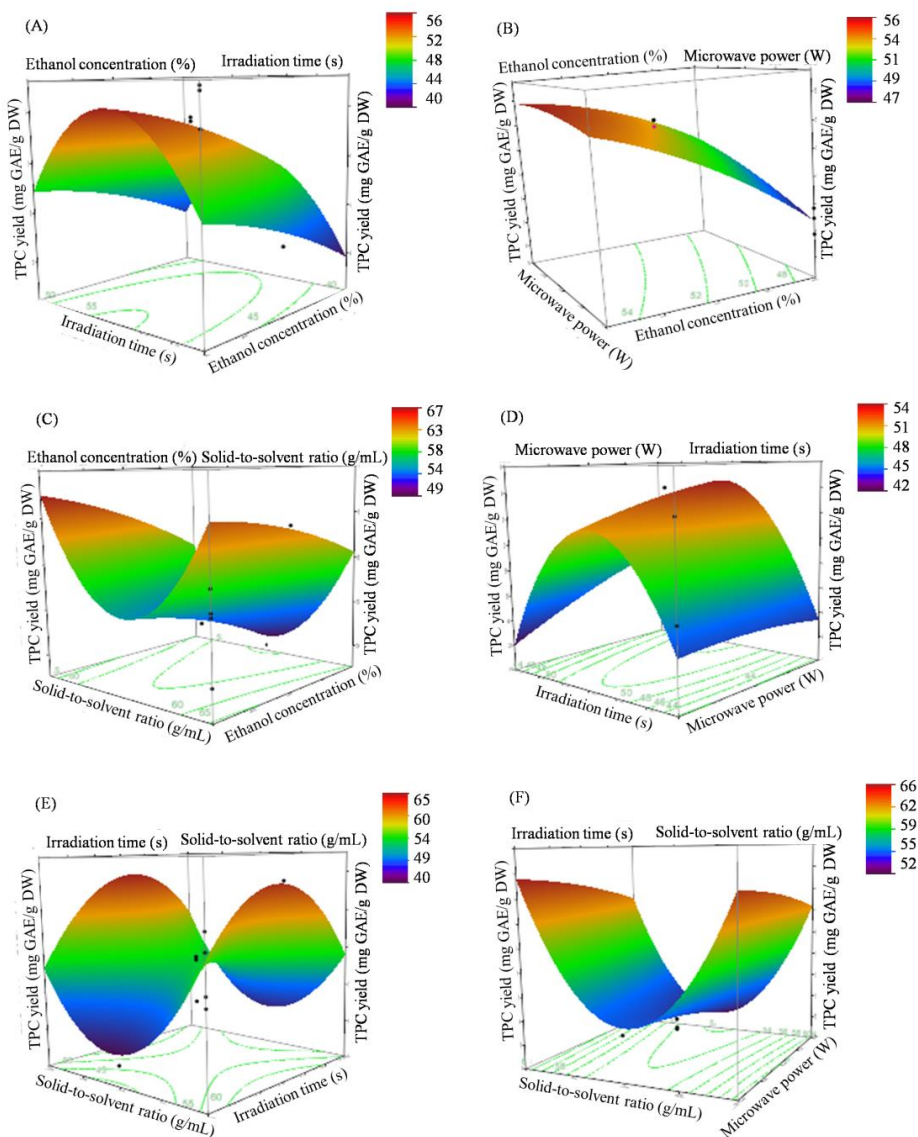
Figure1. Response surface plots for the effect of ethanol concentration and irradiation time (A); Ethanol concentration and microwave power (B); Ethanol concentration and solid-to-solvent ratio (C); Microwave power and irradiation time (D); Irradiation time and solid-to-solvent ratio (E) and Microwave power and solid-to-solvent ratio (F), on the TPC yield from *Lemon verbena* leaves obtained with MAE.

Figure 1A illustrates the interaction between ethanol concentration and extraction time on the TPC yield. Increasing the extraction time from 150 to 180 s induced an increase in the extraction yield, to reach a maximum value of 56.30 mg GAE/g DW with 40% ethanol. Above 180 s, there was a gradual decrease in the response which reaches 46.20 mg GAE/g DW at 210 s (60% ethanol). Indeed, a long exposure to

microwave irradiation could induce the thermos-degradation of the polyphenols (Dahmoune *et al.,* 2014). The extraction rate of *Lemon verbena* leaves polyphenols depends mainly on the extraction time because its linear and quadratic effects were very significant ($< 0.0001^*$) (Table 3). Concerning the solvent concentration, the TPC yield decreases with increasing ethanol concentration. Indeed, the presence of an adequate amount of water in the solvent allows overheating which induce a release of the phenolic compounds (Lefsih *et al.,* 2017).

Figure 1B illustrates the effect of ethanol concentration (X_1) and microwave power (X_3) on the TPC yield. The linear effect of the factors X_1 and X_3 as well as their cross-effect X_1X_3 was highly significant. The maximum extraction rate of 53.80 mg GAE/ g DW was obtained at 40% ethanol and 400 W microwave power, after that it decreases continuously with increasing the ethanol concentration until 80% (400 W).

The interaction effect between ethanol concentration and solid-to-solvent ratio is shown in Figure 1C. As the ethanol concentration increased, the polyphenols yield decreased slightly. For the solid-to-solvent ratio, the TPC yield decreased significantly from 1:20 to 1:30 g/mL. Above this value, the response increased to reach 63.80 mg GAE/g DW at a ratio of 1:40 g/mL, which may be due to the best solubility of polyphenols (Dahmoune *et al.*, 2015).

The linear effect of the factors irradiation time (X_2) and microwave power (X_3) as well as their cross effect (X_2X_3) were highly significant. The response increased when the irradiation time increases from 150 to 180 s and microwave power increases from 200 to 400 W (Figure 1D). Above these values, the extraction efficiency decreased rapidly as the irradiation time increased to minimum value at 210 s. The maximal values of the TPC were obtained at a microwave power of 400 W and an irradiation time of 180 s.

The linear effect of the extraction time (X_2) and solid-to-solvent ratio (X_4) and their interactive effect X_2X_4 were highly significant ($< 0.0001^*$). As shown in Figure 1E, the TPC increased with increasing the extraction time from 150 to 180 s and the solid-to-solvent ratio from 1:30 to 1:40 g/mL to reach a maximum yield of 64.60 mg GAE/g DW. Beyond 180 s the response decreased rapidly.

The cross-effect of microwave power and solid-to-solvent ratio is shown in Figure 1F. The extraction rate, which was maximal (64.40 mg GAE/g DW) at an extraction ratio of 1:20 g/mL and a microwave power of 400 W, decreased significantly with increasing the ratio up to 1:30 g/mL. Above this value, the response increased quickly with increasing the solid-to-solvent ratio to 1:40 g/mL at a microwave power of 400 W. The extraction rate decreased slightly with increasing the microwave power.

Optimal extraction conditions and model validation

The obtained results using the RSM to predict the optimal extraction conditions under microwave irradiation were: 40% (v/v), 188 s, 600 W, and 1:40 g/mL, for ethanol concentration, irradiation time, microwave power, and solid-to-solvent ratio, respectively. The predicted extraction rate under the above conditions was

67.87±1.61 mg GAE/g DW. This value was significantly close to that of the TPC extraction rate calculated experimentally under the optimal conditions which was 67.86±0.92 mg GAE/g DW. These results allowed the validation of the developed regression model for the optimization of process.

Comparison between MAE and ME

Under the optimal conditions, MAE gave a TPC yield of 67.86±0.92[a] mg GAE/g DW, this value was significantly higher ($p < 0.05$) than the yield obtained with ME (34.55±0.90[b] mg GAE/g DW).

The flavonoids yield, obtained with MAE (10.91±0.41[a] mg QE/g DW), was statistically different ($p < 0.05$) than that of ME (1.62±0.03[b] mg QE/g DW). This demonstrates a better efficiency of MAE process compared to ME. The higher TPC and TFC obtained with MAE compared to ME was due to the effect of the microwaves on the plant matrix. Indeed, the MAE guarantees a rapid transfer of energy from the solvent to the plant matrix, inducing a rapid and homogeneous heating, improving TPC recovery (Lefsih *et al.,* 2017). On the other hand, a long extraction time may result in the degradation or conversion of the analytes (Hayat *et al*., 2009; Guemghar *et al.,* 2020).

To the best of our knowledge, there are more studies on the essential oils composition and their biological activities (Bahramsoltani *et al.,* 2018; Djadouni, 2020; Pérez Zamora *et al.,* 2018; Bekara *et al.,* 2020; Mashayekhi-sardoo *et al.,* 2020; Sandner *et al.,* 2020), than on the phenolic compounds of the studied plant. It should also be noted that the works carried out on its phenolic compounds were focused on their characterization (Quirantes-Piné *et al.,* 2009; Quirantes-Piné *et al.,* 2010) as well as on the biological activity of its different extracts or beverages (Moshari-Nasirkandi *et al.,* 2020; Sandner *et al.,* 2020; He *et al.,* 2021). Indeed, few studies were conducted on TPC and TFC. In this study, we tried to compare our results with previous works done on this plant, unfortunately the data were different. This could be due to uncontrolled external factors: agro-climatic conditions, soil composition, harvesting periods, and to controlled factors such as the extraction methods and conditions. Zheng and Wang (2001) reported a content of 1.55±0.1 mg GAE/g of fresh weight from phosphate buffer extract. However, Yoo *et al.* (2008) found higher values of 770.7±2.2 mg GAE/100 g, and 431.60±1.42 mg catechin equivalents/100 g of fresh weight in the hydro-methanolic extract. Dadé *et al.* (2009) reported 1.70±0.19 μmol of caffeic acid equivalent/mg dry matter and 0.50±0.04 μmol of rutin equivalent/mg dry matter from an infusion extract. Recently Jalal *et al.* (2019) obtained 0.86 mg of GAE/mg dry matter and 312.9 mg of rutin equivalent per 100 g of dry matter from an ethanolic extract.

Biological activity

Antioxidant activity

The scientific interest in the antioxidant potential of natural extracts increases continually as they are used in the medical, food and cosmetic fields to replace

synthetic antioxidants known for their toxicity. The results of the antioxidant activities of the optimized extract are shown in Table 4 (a).

According to the results of this study, the *Lemon verbena* extract showed a significantly higher IC_{50} ($p<0.05$) compared to gallic acid IC_{50} for all the antioxidant activities (Figure 2).

In the literature, few works have discussed the antioxidant activity of *Lemon verbena* extracts in terms of IC_{50}. According to the obtained results shown in Table 4 (a), the increase in iron reduction was proportional to the extract concentration. The IC_{50} of the optimized extract was 56.60 ± 2.79^{b} µg/mL, while that of gallic acid was 23.75 ± 2.05^{a} µg/mL. The work carried by Cheurfa and Allem (2016), showed that the hydro-alcoholic macerate of *Lemon verbena* has an antioxidant potential of 6.63 ± 0.10 moles Fe (II)/g of extract. Rezig *et al.* (2019) showed an IC_{50} values for the reducing power of 209.33 μg/mL and 37.33 μg/mL for pure methanolic extract and ascorbic acid, respectively.

Table 4. Biological activitiy of *Lemon verbena* extracts obtained by MAE: antioxidant activity (a) and antibacterial activity (b).

a. Antioxidant activity		
	IC_{50} (µg/mL)	
Sample	**FRAP**	**DPPH**
Extract	56.60 ± 2.79^{b}	139.65 ± 1.44^{b}
Gallic acid	23.75 ± 2.05^{a}	44.16 ± 1.05^{a}

b. Antibacterial activity		
Inhibition diameter (mm)		
	MAE	**ME**
P. aeruginosa	11.50 ± 0.71	/
E. coli	/	/
B. subtilis	5.00 ± 1.41^{a}	4.50 ± 0.71^{b}
S. aureus	13.50 ± 0.71^{a}	5.00 ± 1.41^{b}

MIC and MBC						
	MAE			**ME**		
Strains	**MIC**	**MBC**	**MBC/MIC**	**MIC**	**MBC**	**MBC/MIC**
P. aeruginosa	3.13^{b}	18.75 ± 8.84^{a}	6.00	1.56^{a}	25.00^{b}	16.00
E. coli	3.13^{b}	7.81 ± 6.63^{a}	2.50	1.56^{a}	25.00^{b}	16.00
B. subtilis	1.56^{a}	1.56^{a}	1.00	3.13^{b}	9.37 ± 4.42^{b}	3.00
S. aureus	0.20^{a}	1.56^{a}	8.00	0.20^{a}	25.00^{b}	128.00

S: Sensible, R: Resistant. Values are mean ± standard deviation. Values with different letters (a–, b–, c) were significantly different (Tukey, $p < 0.05$).

The IC_{50} values for the DPPH assay of the optimized extract and gallic acid were 139.65 ± 1.44^{b} µg/mL and 44.16 ± 1.05^{a} µg/mL, respectively. Cheurfa and Allem. (2016) reported an IC_{50} of 23.52 ± 0.04 mg/mL for the ethanolic extract of *Lemon verbena,* compared to that of BHT which was 6.96 ± 0.10 mg/mL. According to Hosseinzadeh and Ebrahimzadeh (2019), the 95% ethanolic extract of *Lemon*

verbena had a high IC_{50} of 21.97±2.4 μg/mL while that of BHA was 53.96±3.16 μg/mL. The pure methanolic extract of *Lemon verbena*, had shown an antiradical activity of 5.78±0.08 μg/mL compared to that of BHT which was 11.5±1.23 μg/mL (Rezig *et al.*, 2019).
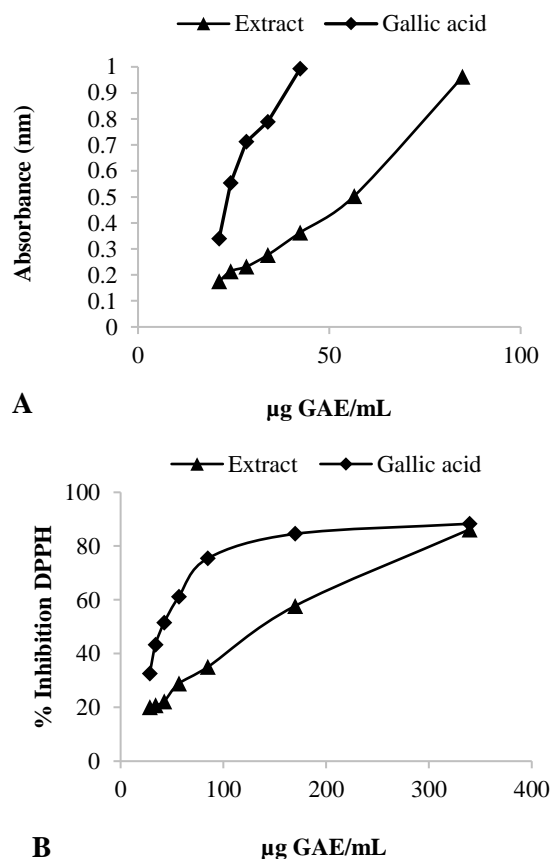

Figure 2. Antioxidant activities of *Lemon verbena* leaves extract obtained by MAE: Ferric reducing antioxidant power (FRAP) (A) and DPPH radical-scavenging assay (B).

Antibacterial activity

The antibacterial activity results were reported in Table 4 (b). According to the antibiogram profile, *S. aureus*, *B. subtilis* and *E. coli* were qualified to be sensitive strains, whereas *P. aerugenosa* was a resistant one. The *Lemon verbena* leaves extracts showed a moderate antibacterial activity, which were concomitant with the findings of Kumar *et al.* (2008). The extract obtained by MAE had the best antibacterial activity comparing to the ME. In fact, it exhibited a considerable antibacterial activity against *P. aeruginosa* and *S. aureus*. However, no activity was shown towards *B. subtilis* and *E. coli* strains. These results corroborate with those of Mirzaie *et al.* (2016). Several studies proved the antibacterial effect of polyphenols on Gram-positive and Gram-negative bacteria. Funes *et al.* (2010)

showed that verbascoside, which is the major polyphenol in this plant, disrupts the structure of the phospholipid membrane. Indeed, the extract obtained with MAE is richer in polyphenols and flavonoids (67.86 ± 0.92^a mg GAE/g DW and 10.91 ± 0.413^a mg QEq/g DW, respectively), than the extract obtained by ME (34.55 ± 0.90^b mg GAE/g DW, and 1.62 ± 0.03^b mg QEq/g DW respectively). This explains the highest antibacterial activity of the MAE extract. According to Tian *et al.* (2009), Polyphenols can alter bacterial cell walls, interact with membrane proteins through hydrogen bonds via their hydroxyl groups. Consequently, this induces changes in membrane permeability and cell destruction, and disrupts the co-aggregation of microorganisms (Naz *et al.,* 2007).

MIC and MBC

The obtained values of MIC and MBC are reported in Table 4 (b). The results show that these values vary depending on the germ and the extraction method. The MIC and MBC values ranged from 0.19 to 3.12 mg/mL and 1.56 to 25.00 mg/mL, respectively. A MBC/MIC ratio less than or equal to 4, indicates the existence of a bactericidal effect of the tested extracts against the different germs (Mamadou *et al*., 2014). The extract obtained by MAE showed a bactericidal effect against *E. coli* and *B. subtilis* with MBC/MIC ratio of 2.5 and 1, respectively, which confers antibiotic power on these strains. However, the macerated extract had a bactericidal effect only on the *B. subtilis* with a MBC/MIC ratio of 3. Comparing the two modes of extraction, it can be seen that the MAE extract has better antibacterial activity with low MBC levels (1.56 to 18.75 mg/mL), compared to the MBC of the extract obtained by ME, with higher concentrations (9.37 to 25.00 mg/mL).This effect could be due to the higher content of phenolic compounds in the extract obtained by MAE (Bouarab-Chibane *et al.,* 2019; Efenberger-Szmechtyk *et al.,* 2021).

According to these results, Gram-negative bacteria are more resistant by registering higher values of MIC and MBC than Gram-positive bacteria; these results were in agreement with those of Efenberger-Szmechtyk *et al.* (2021). This difference is may be due to the distinct cell wall structure between Gram-positive and Gram-negative bacteria. Indeed, a Gram-positive bacterium has a single-layer cell wall structure, whereas Gram-negative bacteria has a multi-layer structure containing an outer cell membrane that forms an impermeable barrier to most molecules (Mamadou *et al.,* 2014).

Conclusions

The *Lemon verbena* is a widely used medicinal plant in traditional medicine for its richness in phenolic compounds. This work aimed to (i) optimize, by the response surface methodology, the microwave-assisted extraction of polyphenols, which enhances the TPC extraction yield in comparison to the conventional maceration method, allowing time and energy saving (both methods were carried out in 188 s and 3 days, respectively), (ii) study the *in vitro* antioxidant activities, of the extract obtained by MAE using DPPH and FRAP methods, (iii) compare both extraction

processes concerning their antibacterial activities. The MAE showed a good antioxidant activity, and the best antibacterial activity compared to ME. These results promote the MAE of the bioactive compounds of this plant at an industrial level, with a view to their use in the medical, cosmetic and food fields.

Acknowledgments

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