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 IC50 of the extract was 139.65±1.44 µg/mL and 56.6 ±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

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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 solvent (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: X1-solvent concentration (%), X2-irradiation time (s), X3-microwave power (W) and X4-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):

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \sum_{j>i}^{k} \beta_{ij} X_i X_j + E \]  

where \( \beta_0 \) is the constant coefficient of the model, \( \beta_i \), \( \beta_{ij} \) and \( \beta_{ij} \) 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).
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, 2020). 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.

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

<table>
<thead>
<tr>
<th>Particle size (µm)</th>
<th>Type of solvent</th>
<th>Solvent concentration (%)</th>
<th>Irradiation time (s)</th>
<th>Microwaves power (W)</th>
<th>Solid-to-solvent ratio (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>EtOH</td>
<td>54.00±1.18 g/g</td>
<td>90</td>
<td>54.40±1.25 g/g</td>
<td>1:15</td>
</tr>
<tr>
<td>250</td>
<td>MeOH</td>
<td>57.60±1.40 g/g</td>
<td>120</td>
<td>54.70±1.16 g/g</td>
<td>1:20</td>
</tr>
<tr>
<td>500</td>
<td>Acetone</td>
<td>52.76±1.40 g/g</td>
<td>150</td>
<td>59.13±1.59 g/g</td>
<td>1:25</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>46.04±1.10 g/g</td>
<td>60</td>
<td>55.71±1.26 g/g</td>
<td>1:30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>210</td>
<td>55.18±1.70 g/g</td>
<td>1:55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>240</td>
<td>50.23±1.15 g/g</td>
<td>1:40</td>
</tr>
</tbody>
</table>

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

**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 irradiation time, and reached its maximum after 180 s. Beyond this value, the extraction yield decreased progressively as the irradiation time was prolonged (Dahmoune et al., 2014). Thus, 180 s was the optimal point used for the single-factor tests, and the range 150-210 s was used to perform the RSM tests.

**Effect of ethanol concentration**

The TPC yield increased as the ethanol concentration increased from 40 to 80%. The 60% of ethanol concentration was set for the single-factor experiments, and the range 40-80% was chosen for 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 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 (X1, X2, X3 and X4, 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 X1, X2, X3, quadratic effects X2^2 and X3^2 as well as interactions X1X5, X2X3 and X2X4 were highly significant (< 0.0001*). Solid-to-solvent ratio X4, quadratic effects X1^2, X3^2, interactions X1X2, X1X4 and X3X4 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).
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).

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

<table>
<thead>
<tr>
<th>Run</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>$X_4$</th>
<th>Recovery of TPC (mg GAE/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>1</td>
<td>80 (+1)</td>
<td>150 (-1)</td>
<td>200 (-1)</td>
<td>40 (+1)</td>
<td>46.0±0.90$^{abc}$</td>
</tr>
<tr>
<td>2</td>
<td>60 (0)</td>
<td>180 (0)</td>
<td>400 (0)</td>
<td>30 (0)</td>
<td>54.2±0.20$^{abc}$</td>
</tr>
<tr>
<td>3</td>
<td>80 (+1)</td>
<td>210 (+1)</td>
<td>600 (+1)</td>
<td>20 (-1)</td>
<td>54.8±1.12$^{bcde}$</td>
</tr>
<tr>
<td>4</td>
<td>40 (-1)</td>
<td>210 (+1)</td>
<td>600 (+1)</td>
<td>40 (+1)</td>
<td>62.6±1.31$^{bcde}$</td>
</tr>
<tr>
<td>5</td>
<td>80 (+1)</td>
<td>210 (+1)</td>
<td>200 (-1)</td>
<td>20 (-1)</td>
<td>47.0±1.41$^{kl}$</td>
</tr>
<tr>
<td>6</td>
<td>60 (0)</td>
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<td>400 (0)</td>
<td>20 (-1)</td>
<td>64.4±2.44$^{a}$</td>
</tr>
<tr>
<td>7</td>
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<td>200 (-1)</td>
<td>40 (+1)</td>
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<tr>
<td>8</td>
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<td>600 (+1)</td>
<td>20 (-1)</td>
<td>59.2±2.26$^{abc}$</td>
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<td>200 (-1)</td>
<td>20 (-1)</td>
<td>59.8±0.41$^{bcde}$</td>
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<tr>
<td>11</td>
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<td>150 (-1)</td>
<td>200 (-1)</td>
<td>20 (-1)</td>
<td>52.9±3.25$^{ghijkl}$</td>
</tr>
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<td>40 (+1)</td>
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<tr>
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<td>30 (0)</td>
<td>42.7±2.12$^{p}$</td>
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<tr>
<td>24</td>
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<td>180 (0)</td>
<td>400 (0)</td>
<td>30 (0)</td>
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<tr>
<td>25</td>
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<td>600 (+1)</td>
<td>40 (+1)</td>
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<tr>
<td>26</td>
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<td>27</td>
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<td>600 (+1)</td>
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<td>400 (0)</td>
<td>30 (0)</td>
<td>52.6±1.37$^{ghijkl}$</td>
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<td>29</td>
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<td>200 (-1)</td>
<td>40 (+1)</td>
<td>59.2±0.85$^{bcde}$</td>
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<td>30</td>
<td>40 (-1)</td>
<td>210 (+1)</td>
<td>200 (-1)</td>
<td>20 (-1)</td>
<td>56.8±1.36$^{bcde}$</td>
</tr>
</tbody>
</table>

Table 2: 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 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):

\[
Y(\text{TPC}) = 53.21 - 3.47X_1 + 1.23X_2 + 1.25X_3 - 8.51X_2^2 + 11.13X_4^2 + 1.2X_1X_3 + 1.49X_2X_3 + 1.67X_2X_4
\]

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).
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 (X₁) and microwave power (X₃) on the TPC yield. The linear effect of the factors X₁ and X₃ as well as their cross-effect X₁X₃ 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 (X₂) and microwave power (X₃) as well as their cross effect (X₂X₃) 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 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 mg GAE/g DW, this value was significantly higher (p < 0.05) than the yield obtained with ME (34.55±0.90 mg GAE/g DW).

The flavonoids yield, obtained with MAE (10.91±0.41 mg QE/g DW), was statistically different (p < 0.05) than that of ME (1.62±0.03 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 µg/mL, while that of gallic acid was 23.75±2.05 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 activity of *Lemon verbena* extracts obtained by MAE: antioxidant activity (a) and antibacterial activity (b).

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC$_{50}$ (µg/mL)</th>
<th>FRAP</th>
<th>DPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>56.60±2.79$^b$</td>
<td>139.65±1.44$^b$</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>23.75±2.05$^a$</td>
<td>44.16±1.05$^a$</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibition diameter (mm)</th>
<th>MAE</th>
<th>ME</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>11.50±0.71</td>
<td>/</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>5.00±1.41$^b$</td>
<td>4.50±0.71$^b$</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>13.50±0.71$^a$</td>
<td>5.00±1.41$^b$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC</th>
<th>MBC</th>
<th>MBC/MIC</th>
<th>MIC</th>
<th>MBC</th>
<th>MBC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>3.13$^b$</td>
<td>18.75±8.84$^a$</td>
<td>6.00</td>
<td>1.56$^a$</td>
<td>25.00$^b$</td>
<td>16.00</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3.13$^b$</td>
<td>7.81±6.63$^a$</td>
<td>2.50</td>
<td>1.56$^a$</td>
<td>25.00$^b$</td>
<td>16.00</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>1.56$^a$</td>
<td>1.56$^a$</td>
<td>1.00</td>
<td>3.13$^b$</td>
<td>9.37±4.42$^b$</td>
<td>3.00</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.20$^a$</td>
<td>1.56$^a$</td>
<td>8.00</td>
<td>0.20$^a$</td>
<td>25.00$^b$</td>
<td>128.00</td>
</tr>
</tbody>
</table>

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 µg/mL and 44.16±1.05 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).

![Antioxidant activities of Lemon verbena leaves extract obtained by MAE: Ferric reducing antioxidant power (FRAP) (A) and DPPH radical-scavenging assay (B).](image)

**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. aeruginosa 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 mg GAE/g DW and 10.91±0.41 mg QEq/g DW, respectively), than the extract obtained by ME (34.55± 0.90 mg GAE/g DW and 1.62±0.03 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.

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