# *Phenolic content and antioxidant activities of* Vitis vinifera *L. leaf extracts obtained by conventional solvent and microwave-assisted extractions*

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ORIGINAL PAPER

# Phenolic content and antioxidant activities of *Vitis vinifera* L. leaf extracts obtained by conventional solvent and microwave-assisted extractions

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## Abstract

Grapevine leaves are used in the cuisines in the countries surrounding the Mediterranean and in folk medicine. They contain a huge amount of phenolic constituents. To optimize the extraction of bioactive compounds present in grapevine leaves, two methods of extraction were compared: conventional solvent extraction (CSE) and microwave-assisted extraction (MAE). The optimal extraction conditions in term of total phenolic content (TPC) were determined using a Box–Behnken design (BBD) from leaves of *Vitis vinifera* L. cv. Le tizourine Bou Afraraet. Optimal extraction conditions for CSE was 29% concentration of ethanol (v/v), 30.96 min extraction time, and 72:1 liquid-to-solid ratio (mL:g), at 37.5 °C. For MAE it was 34% concentration of ethanol(v/v), 474 W microwave power, 47 s irradiation time and 40:1 liquid-to-solid ratio (mL:g). Both extracts obtained by optimized MAE and CSE processes were compared in terms of total flavonoid content (TFC) and antioxidant activities (DPPH, ORAC, PFRAP and ICA). The MAE extract exhibited highest phenolic content and antioxidant activities. Moreover, phenolic compounds from leaves of *Vitis vinifera* L. cv. Ahmar-Bou-Amar were extracted following MAE optimal conditions and compared to Le tizourine Bou Afraraet extract. In addition to TPC and antioxidant activities, phenolic constituents were determined using HPLC–DAD-IT-MS and UPLC-QqQ-MS/MS. A total of sixteen phenolic compounds were identified and quantified including two phenolic acids, seven flavonols, three flavanols and four stilbenes. The obtained data revealed that the leaves of two native Algerian grapevines are rich in bioactive constituents, and can constitute by-products with added-value for food supplements.

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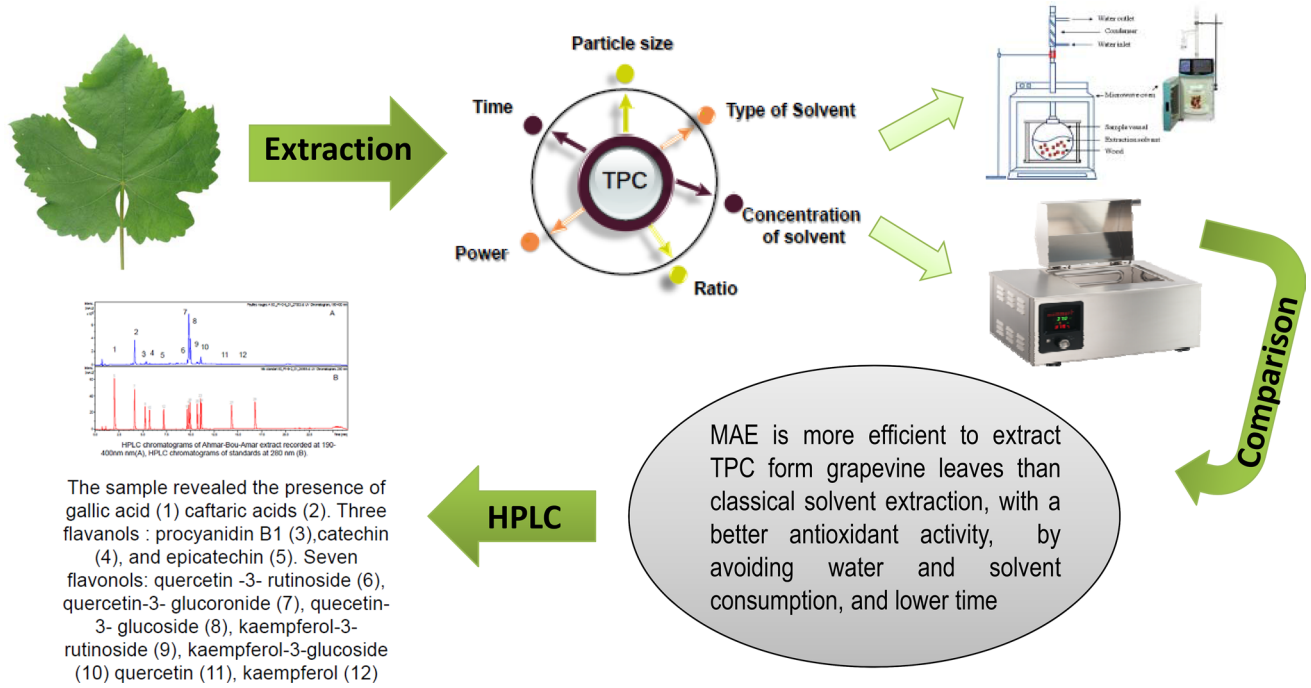
**Graphic abstract**




**Keywords** Grapevine leaves · Polyphenols · Microwave-assisted extraction · Optimization · Antioxidant activity

## Introduction

Plant-based foods and their derivative products, as complementary food, can be beneficial to moderate the oxidative damage involved in different chronic disease [1, 2]. Phenolic compounds thanks to their antioxidant properties are considered to be key source of these benefits [2]. The importance and relevance of polyphenols from plant leaves as powerful antioxidant compounds has been demonstrated by several previous studies [3, 4]. Among cultivated plants, grapevine occupies an important place around the Mediterranean region that is used for production of table grapes, dried fruits, grape juice, and wine [5]. In Algeria, viticulture was developed before the French colonization with an estimated area of three thousand hectares. It is based on native grape varieties and those introduced from the Mideast by the Turks [6]. Grapevines are commonly cultivated for its berries and the leaves are often not considered for food industry, even if sometimes they are used in food gastronomy and traditional medicine [7]. As grapevine leaves are enriched in polyphenols and already used in food, they could represent a low-cost source of functional ingredients. The use of by-products coming from plants with a food application has undeniable advantages. To develop such approach, it is relevant to follow an environmentally friendly transformation path. In this context, several applications have already been carried out in other fields either to take advantage of the ingredients or to develop new products [8]. For an optimal valorization of by-products, the control of the extraction process conditions as well as the behaviors of the food matrix is necessary. These last 10 years, numerous novel extraction procedures have been initiated, most of which were claimed to improve efficiency, extract quality, extraction time and solvent consumption [9]. Among these emerging and promotional technologies, the benefits of application of pulsed electrical technologies and ultrasound to improve the recovery of phytochemicals have been reported [10]. Usually, five steps are set out for the recovery of products of interest from plant matrices: grinding, extraction, isolation, purification and concentration of the desired molecules like antioxidants [11]. To study phenolic compounds of grapevine leaves, their extraction has been often carried out in the last decade using conventional solid/liquid processes [12, 13]. Microwave-assisted extraction (MAE) applied to plant phenolic compounds presents several advantages in comparison to conventional extraction procedures such as solvent and time reduction. Nevertheless, due to the diversity of the plant material, the extraction conditions may be optimized for each vegetal matrix. Among the strategies that can be used for the optimization

of experimental conditions, response surface methodology (RSM) is a common one [14]. To our knowledge, no previous investigations were published concerning the optimization of the extraction of phenolic compounds from grapevine leaves by MAE. In addition, only few studies reported the optimization of conventional solvent extraction (CSE) from grape by-products. The main goal of this study was to obtain a better survey into optimization of extraction conditions of phenolic compounds from grapevine leaves. Firstly, phenolic compounds from leaves of the Algerian grape *Vitis vinifera* L. cv. Le Tizourine Bou Afraraet were extracted using two different extraction methods (CSE and MAE). Secondly, the best method of extraction (MAE) was applied to another Algerian cultivar *Vitis vinifera* L. cv. Ahmar-Bou-Amar. The two native varieties were compared in term of phenolic content and antioxidant properties. In addition, their phenolic profiles were determined using UHPLC-DAD-IT-MS and UHPLC-QqQ-MS/MS analyses.

## Materials and methods

### Chemicals

Carbonate ($Na_2CO_3$), Folin–Ciocalteu reagent, 1-diphenyl-2-picrylhydrazyl radical (DPPH), aluminum chloride, 2,2 -azobis(2-amidinopropane) dihydrochloride (AAPH),Iron(II) sulphate ($FeSO_4$), phosphate buffer (0.1 M, pH 7.4), ferric chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), trichloroacetic acid, potassium ferricyanide, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid sodium salt (ferrozine), 3′,6′-dihydroxyspiro [isobenzofuran-1[3H],9′[9H]- xanthen]-3-one(fluorescein), and L-ascorbic acid were provided by Sigma-Aldrich (St. Louis, MO, USA). Concerning phenolic compounds (catechin, epicatechin, gallic acid, caftaric acid, resveratrol, and piceid), they were provided by Sigma-Aldrich (St. Louis, MO, USA);quercetin and kaempferol derivatives were provided by Extra synthese (France). Methanol and ferrous sulfate were supplied by Prolabo (France). Ethylene diamine tetraacetic acid (EDTA) was supplied from Fluka. Ultrapure water was obtained using an Elga water purification system (Bucks, UK, 18 MΩ).

### Plant material

Grapevine leaves (*Vitis vinifera* L. cv. Le Tizourine Bou Afraraet and cv. Ahmar-Bou-Amar) were harvested at random, from several vine trees at maturity from wild lands in September, 2017 in the locality of Kendira province of Algeria (North East of Algeria-Bejaia; latitude 36° 31′ 58; longitude 5° 3′ 46, altitude 945 m), a region with a Mediterranean climate. All samples were placed directly in rigid boxes. Then, leaves were washed well with tap water, drained and dried in a ventilated darkroom at ambient temperature (20 °C) for about 20 days. Once dried, the leaves were grinded to get a fine powder with an electric grinder (IKA model-A11, Staufen, Baden-, Germany). The powder was sifted through an electric sieve (Retsch As 200, Haan, Germany) with a downward porosity: 125 µm, 250 µm and 500 µm and then kept at 20 °C far from light until required.

### Experimental work

Various extraction procedures were developed to take out phytochemicals from plant material. These methods are based on the extracting power of various solvents and the exposure to heat and/or stirring [15]. To compare a conventional method (CSE) and emerging method (MAE) based on the maximum TPC present in the obtained extracts, the optimization of conventional solvent extraction (CSE) and microwave assisted extraction (MAE) were performed on leaves of *V. vinifera* cv. Le tizourine Bou Afraraet, a white vine variety. The experimental design constituted of two main parts. Firstly, single-factor experiments were conducted to define the proper range of conditions for phenolic extraction of grape leaves keeping the variable that is not studied constant. For this step, the variable considered for CSE extraction were: particle size (µm), type of solvent (ethanol, methanol, acetone and distilled water), ethanol concentration (%, v/v), temperature (°C), extraction time (min), and solvent-to-solid ratio (mL/g). In a similar way, six variables were considered for MAE: particle size (µm), type of solvent (ethanol, methanol, acetone and distilled water), ethanol concentration (%, v/v), microwave power (W); irradiation time (s); and solvent-to-solid ratio (mL/g). All trials were repeated three times. Secondly, the CSE and MAE performance study was obtained by the response surface methodology (RSM) based on Box–Behnken Design (BBD), using the Software JMP (SAS Institute, Brie Comte Robert, France) for data analysis and model building. Based on the single-factor analyses, the factors having the strongest influence were selected. The RSM processes based on a Box–Behnken Design (BBD) were performed for CSE and MAE. Four variables were selected to optimize CSE namely $X_1$: concentration of ethanol (%, v/v); *X2*: temperature (°C); *X3*: time (min); and $X_4$: solvent-to-solid ratio (mL/g). Similarly for the CSE, four variables were used $X_1$: ethanol concentration (%, v/v); *X2*: microwave power (W); *X3*: irradiation time (s); and $X_4$: solvent-to-solid ratio (mL/g). All experimental results were obtained from a 27-run-experiment (see Tables S1 and S2), which were repeated three times for each assay.

#### Conventional solvent extraction

Grapevine leaves of *V. vinifera* cv. Le tizourine Bou Afraraet were submitted to CSE following the procedures developed by Spigno et al. [16] with slight modifications. In a thermostatic water bath (model WNB22, Memmert, Frankfurt, Germany), 1 g of powder was mixed with the suitable solvent volume, and the process is started following the conditions indicated in Table S1. At the end of the processes, the extract was filtered throw a filter paper (Whatman N°1, USA) then stored at 4 °C. Triplicate assays have been carried for each extraction.

Using the data collected in the single-factor analyses, Box–Behnken Design protocol with the four factors and three levels was used to investigate and optimize the impact of process variables: ethanol concentration (%, v/v) (20–60), temperature (°C) (20–60), time (min) (20–60), and liquid-to-solid ratio (mL:g) (60–100).

#### Microwave assisted extraction

A domestic microwave apparatus (Maxipower model MAXMO23S, Shenzhen, Guangdong, China) was used for extraction of phenolics from grapevine leaves of *V. vinifera* cv. Le tizourine Bou Afraraet. One g of leaf powder was subjected to microwave irradiation at different frequencies and intervals as specified in Table S2. The extract was filtered through a filter paper (Whatman N°1, USA), and then collected and gathered at 4 °C. Extractions were performed three times. The optimal ranges of ethanol concentration (%, v/v) (20–60), microwave power (W) (300–700), irradiation time (s) (30–90) and solvent-to-solid ratio (mL:g) (20–60) were chosen from the preliminary experiments without using mathematical models.

This model was used to generate the optimal conditions for extraction and better examine and clarify the relevance of the method according to the following equation:

$$Y = B_0 + \sum_{i} = 1^4 B_i X_i + \sum_{i} = 1^3 \sum_{j} = i + 1^4 B_{ij} X_i X_j + e_i$$

where Y indicates the response; $B_0$ is a constant coefficient; $B_i$ represents the linear coefficient, $B_{ii}$ is a quadratic coefficient and $B_{ij}$ is an interactive coefficient. $X_i$ and $X_j$ symbolize the variables being independent and $e_i$ is the error.

### Chemical analyses

#### UHPLC-DAD-IT-MS analysis

The chromatography apparatus was an Agilent 1290 Series (Agilent Technologies, Santa Clara, CA, USA) dotted with an auto sampler module, a binary pump with a degasser, a column heater/selector and a UV/vis diode array detector (DAD). We used a column with the following characteristics: Zorbax SB-C18 (100 mm × 2.1 mm, 1.8 µm) with a guard column (Agilent Technologies, Santa Clara, CA, 2.1 mm × 5 mm i.d). Parameters of elution consisted of a flow set on 0.4 mL/min with acidified water (0.1% formic acid; v/v) like solvent A and acetonitrile acidified (0.1% formic acid; v/v) as solvent B using the next gradient: 1 to 32.5% B (0–17 min), 32.5% B (17–19 min), 32.5 to 95% B (19–22 min), 95% B (22–24 min), 95 to 5% B (24–26 min). An Esquire 6000 ion trap mass spectrometer (Bruker-Daltonics, Billerica, MA) with an electrospray ionization source was linked to the UHPLC. The MS method was performed with the following parameters: negative mode (range of *m/z* 130–950); gas for drying (nitrogen), 10 L/min; nebulizer pressure, 40 psi; temperature, 350 °C; capillary voltage, 4500 V; capillary exit voltage, − 133.3 V; skimmer voltage, − 66 V; trap drive, 41.2. Extracts of grapevine leaves were assayed by dissolving powders in a methanol-water mixture (1:1, v/v) at 50 mg/mL, filtered on 0.45 µm poly tetra fluoro ethylene (PTFE) and achieved in three assays. Data were analyzed using DAD chromatograms obtained at 190 and 400 nm. Calibration and equation curves were obtained by using standards in independent quintuplicate at several concentrations (0.9–50 µg/mL). Concentrations were further calculated as mg per g of dry weight (mg/g DW) based on three replicates.

#### UHPLC-QqQ-MS/MS analysis of stilbenes

Extracts were analyzed for their content in stilbenes by a previously developed method by [17] with a 1260 Infinity UPLC (Agilent Technologies, Courtaboeuf, France) coupled to a 6430 triple quadrupole mass spectrometer (Agilent Technologies, Courtaboeuf, France). We injected one microliter of the sample into an Agilent Zorbax SB-C18 (100 mm × 2.1 mm, 1.8 µm) thermostated at 40 °C. The acidified water (0.1% formic acid, v/v) was used as solvent A and acidified acetonitrile (0.1% formic acid, v/v) as solvent B. The parameters of elution consisted of a flow fixed on 0.4 ml/min following the gradient:18% B (0–3.5 min), 18 to 33% B (3.5–6.5 min), 33 to 40% B (6.5–12 min), 40 to 95% B (12–13 min), 95% (13–16 min), 95 to 5% B (16–16.5 min). The limitations were set as follow: dry temperature, 350 °C; nebulizer pressure, 15 psi; dry gas, 10 L/min; capillary voltage, + 3 kV. Standards of *trans*-resveratrol and *trans*-piceid ranging from 0.04 to 6.25 µg/mL were used for the calibration curve. *cis*-Resveratrol and *cis*-piceid were indicated as equivalents of *trans*-resveratrol and *trans*-piceid, respectively. All samples were analyzed three times.

#### Total polyphenols analysis

Total phenolic content (TPC) of each vine leave extract were assayed using the Folin–Ciocalteu method [18]. Appropriate dilutions of samples were mixed with Folin–Ciocalteu reagent (1/10), the combination was kept at ambient temperature for 2 min, then we added 80 µL of carbonate of sodium (75 g/L), and the composite was incubated for 15 min at 50 °C. The absorbance was recorded at 760 nm employing an UV–Visible light spectrophotometer (UV–VIS Spectrophotometer UV-1800, Shimadzu, Kyoto, Japan). The expression of the data was enunciated in mg gallic acid equivalents per gram of extract (mg GAE/g). Triplicate assays have been realized for all extracts.

#### Total flavonoid

Total flavonoid contents (TFC) were estimated using the method reported by Djeridane, Yousfi, Nadjemi, Boutassouna, Stocker and Vidal [19]. Thus, we added 1 mL of aluminum chloride prepared at 2% in methanol to 1 mL of samples. Before analysis, we left the two substances act for 15 min in the dark. Samples absorbance was determined at 430 nm versus the prepared blank using UV–vis light spectrophotometer (UV–VIS Spectrophotometer UV-1800, Shimadzu, Kyoto, Japan). The expression of achieved data was given in mg quercetin equivalent per g of extract (mg QE/g). Each vine leave extract has been tested three times.

#### Antioxidant activities

**DPPH assay** The grapevine leaves extracts free antioxidant power was assessed by the method described by Blois [20]. Briefly in a black 96-well plate, 50 µL of leaf extract were mixed with 150 µL of DPPH dissolved in methanol (200 µM). After 20 min far from light and at room temperature, the reduction in DPPH· was estimated by determining absorbance at 520 nm. The anti-radical capacity of the concerned extracts was converted to mg of Trolox equivalent per g of extract (mg TE/g). All samples were processed three times.

**Test of ORAC** The Oxygen Radical Absorbance Capacity (ORAC) assay was realized like outlined by Ou, Hampsch-Woodill and Prior [21] modified by Davalos, Gomez-Gordoves and Bartolomé [22]. To 30 µL of extract sample, standard (Trolox) or blank sample (phosphate buffer) placed into black 96-well plates, were added 180 µL of fluorescein prepared at 70 nM. In the plate reader at 37 °C, the all was incubated for 10 min, then 90 µL of AAPH prepared at 12 mM were added at each cycle. The absorbance reading was made on excitation and emission wavelength of 485 nm and 530 nm, respectively. Values of ORAC were communicated as mg of Trolox equivalents per g of extract (mg TE/g) using a curve of the selected standard conducted in same conditions. All grapevine leaves extracts were assayed in triplicate.

**PFRAP test** The Potassium Ferricyanide Reducing Antioxidant Power of the vine leaves extracts was studied using the procedure of Oyaizu [23]. Briefly, a mixture containing 10 µL of sample or standard (ascorbic acid) and 30 µL of phosphate buffer (0.2 M, pH 6.6) and 30 µL of potassium ferricyanide (1%, w/v) was prepared. The suspension was kept for 20 min at 50 °C, after which 30 µL of trichloroacetic acid (10%, w/v) was put, then we added 100 µL of water and finally 20 µL of ferric chloride (1%, w/v). Absorbance was read at 700 nm after keeping the solution to react for 30 min. We expressed the results as mg ascorbic acid equivalent per g of extract (mg AAE/g). All vine leaves extracts were assessed three times.

**ICA test** The Iron Chelating Activity (ICA) of grapevine leaves extracts was measured by the method described by Dinis, Madeira and Almeida [24]. Thus, 40 µL of the diluted extract and 60 µL of $FeSO_4$ (0.2 mM) were mixed. Then we add 80 µL of ferrozine (2 mM). ICA values were estimated by determining absorbance at 562 nm, after a waiting time of about 10 min. A curve of calibration with a range of EDTA chelating agent concentrations was used and the results were enounced as mg EDTA equivalents/g of extract (mg EDTA/g). All grape leaves samples were investigated in triplicate.

#### Statistical analysis

The preliminary tests results and the data comparing the CSE and MAE of the two varieties of grapevine leaves were investigated using the ANOVA statistical model at a level of signification of 5% ($p < 0.05$) with the help of the STATISTICA 5.5 software. The analysis of the adjustment of the mathematical models was performed with the software JMP software (version 7.0, SAS) on bases of the following parameters: the lack of adjustment, the root-mean-square error (RMSE), and the correlation coefficient $R^2$.

## Results and discussion

### Experimental plans assessment

#### Optimization of CSE conditions

The experimental procedure and TPC values from grapevine leaves (Le Tizourine Bou Afraraet cultivar) are summarized in Table S1 which recapitulates the 27 trials. TPC values are

ranged from 32.5 to 58.8 mg GAE/g. To explain the connection between TPC and operational conditions, the second degree equation was obtained; omitting the insignificant terms ($P > 0.01$), the final predictive equation for CSE was given below:

$$\begin{aligned} Y(TPC) = {} & 47.40 - 4.77X_1 + 0.095X_2 - 0.55X_3 - 2.86X_4 \\ & + 2.57X_1X_2 - 6.77X_1X_3 - 3.04X_2X_3 \\ & + 2.58X_1X_4 + 1.88X_2X_4 + 2.75X_3X_4 \\ & - 2.85X_1^2 - 2.44X_2^2 + 2.61X_3^2 - 7.49X_4^2 \end{aligned} \quad (1)$$

where $X_1$ concentration of ethanol (%, v/v); *X2* degree of temperature (°C); *X3* time (min); and $X_4$ liquid-to-solid ratio (mL:g).

The variance analysis (ANOVA), the fit goodness and the model adequacy were given in Table 1. Coefficient of determination ($R^2$) value close to 1.0 indicates a good fit. The $R^2$ and the adjusted coefficient of determination ($R^2$-adjusted, whose value should not be far from $R^2$) of the model were 0.98 and 0.94, respectively. These values indicate a good fit between experimental and predicted TPC values, and the high significance of the model. The *F*-value rate for the lack of fit is not significant. The root-mean-square error (RMSE) low value (1.53) confirms that this model is significant to predict the extraction of phenolics from grapevine leaves. Finally, all the interaction parameters ($X_1X_2$, $X_1X_3$, $X_2X_3$, $X_1X_4$, $X_2X_4$, $X_3X_4$), and their quadratic coefficients ($X_1^2$, $X_2^2$, $X_3^2$, $X_4^2$) of the model are significant($p < 0.05$)indicating that each factor affects one another.

The regression analysis of the data reveals that liquid-to-solid ratio and concentration of ethanol have a notable impact on the response function in linear forms. This result confirms the importance of solvent concentration in comparison to other factors [25, 26]. While no previous information relating to the optimization of extraction conditions for grapevine leaves was found, studies on other parts of the plant (seeds and skin of the fruit) have shown the influence of the liquid-to-solid ratio on the TPC extraction [27]. The three-dimensional response surfaces were plotted from Eq. (1) to study the effects of each variable and their mutual interactions concerning the TPC value (Fig. 1). The results indicate that TPC value is strongly dependent on the variables $X_1$ and $X_4$ (concentration of ethanol and liquid-to-solid ratio, respectively). The greatest value was obtained with followed parameters: 40 min extracting time; concentration of ethanol ranged between 30 and 45% (v/v); 80:1 liquid-to-solid ratio.

The TPC values depend strongly of concentration of ethanol as linear and quadratic effects were highly significant (Table 1, Fig. 1a). In agreement with single-factor experiments, the TPC value suggests that phenolics are more soluble in 40% concentration of ethanol. For the highest concentration of ethanol (60%), the TPC value decreased.

**Table 1** Analysis of mean square deviation of the quadratic model terms applied to the experimental values of total phenolic yields obtained with conventional solvent extraction

| Source | Sum of squares | df[a] | F value | P value > F | |
|---|---|---|---|---|---|
| Model | 1173.14 | 14 | 35.60 | < 0.0001 | Significant |
| $X_1$—ethanol | 273.79 | 1 | 116.33 | < 0.0001 | |
| $X_2$—temperature | 0.110 | 1 | 0.047 | 0.832 | |
| $X_3$—time | 3.663 | 1 | 1.556 | 0.236 | |
| $X_4$—ratio | 98.728 | 1 | 41.950 | < 0.0001 | |
| $X_1X_2$ | 26.471 | 1 | 11.248 | 0.006 | |
| $X_1X_3$ | 183.330 | 1 | 77.898 | < 0.0001 | |
| $X_2X_3$ | 37.088 | 1 | 15.759 | 0.002 | |
| $X_1X_4$ | 26.780 | 1 | 11.379 | 0.006 | |
| $X_2X_4$ | 14.213 | 1 | 6.039 | 0.030 | |
| $X3X_4$ | 30.305 | 1 | 12.877 | 0.004 | |
| $X_1^2$ | 43.485 | 1 | 18.477 | 0.001 | |
| $X_2^2$ | 31.991 | 1 | 13.593 | 0.003 | |
| $X_3^2$ | 36.494 | 1 | 15.506 | 0.002 | |
| $X_4^2$ | 299.234 | 1 | 127.145 | < 0.0001 | |
| Residual | 28.242 | 12 | | | |
| $R^2$ | | | | 0.98 | |
| Adjusted $R^2$ | | | | 0.94 | |
| Lack of fit | 22.262 | 10 | 0.745 | 0.695 | Not significant |
| Pure error | 5.979 | 2 | | | |
| Cor total | 1201.38 | 26 | | | |

[a]*df* degrees of freedom

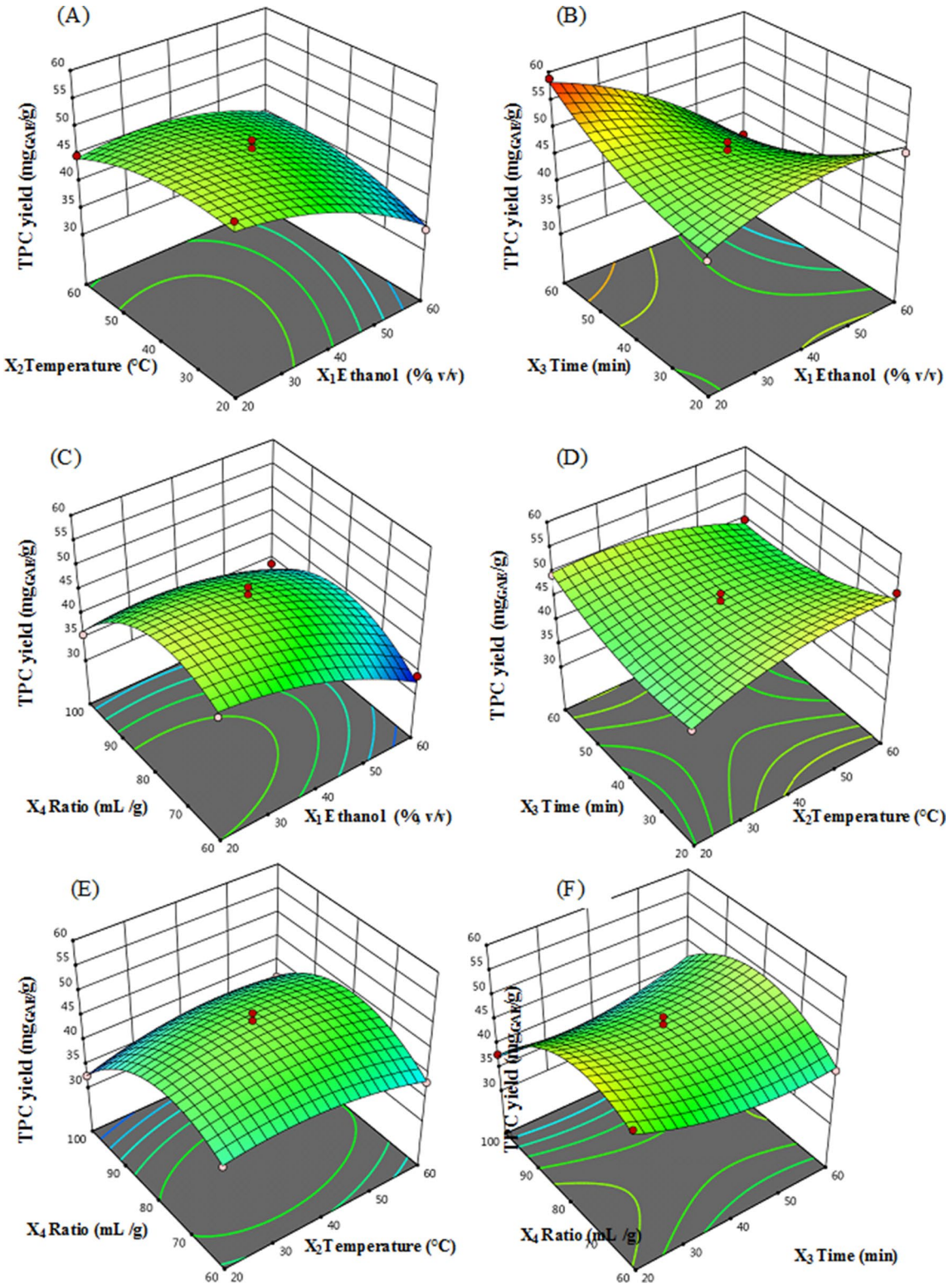



**Fig. 1** Response surface analysis of the effect of conventional solvent extraction on TPC value of grapevine leaves extracts: **a** temperature and ethanol concentration, **b** time and concentration of ethanol, **c** solid-to-liquid ratio and ethanol concentration, **d** time and temperature, **e** solid-to-liquid ratio and temperature, and **f** solid-to-liquid ratio and time

This observation could be due to the polyphenols diffusion coefficient that decreases when the concentration of ethanol increases as mentioned by Muñiz-Márquez et al. [28]. Additional concentration of ethanol and time caused negative effects resulting in a curvilinear decrease of the TPC values (Fig. 1b). Lower TPC value was obtained for 60 min and 60% concentration of ethanol. These findings were in agreement with that of Spigno et al. [16] which underlined the influence of ethanol on the extraction of phenolics from pomace. The TPC values are also positively correlated to the liquid-to-solid ratio as indicated by linear and quadratic effects (Table 1), and the response surface curves (Fig. 1c, e, f). This observation is consistent with the mass transfer which is mainly governed by the gradient of concentration between the solid and the bulk of the liquid and by the increase with the solvent-to-solid ratio [29]. As previously observed, the temperature is positively correlated to the extraction of phenolics [16, 30]. but with a less important effect than concentration of solvent in the range of temperature used (Table 1). Additionally, its quadratic effects with the other factors were significant (Table 1). High temperature could cause softening of the plant tissue, weakening of the integrity of cell walls, and a hydrolyze of the phenol–protein and phenol-polysaccharide bonds, as well as improve the solubility of phenolics, which increases their diffusion in the solvent [31]. As observed in Fig. 1d, high temperatures and extraction time are positively correlated to the TPC value. Indeed, the diffusion of solid molecules in the solvent is improved by the increase of the contact time [32]. and also by raising the temperature of extraction [33]. This observation is in agreement with the findings described by Kovačević et al. [34], on a work carried out on *Stevia rebaudiana Bertoni* leaves which demonstrated that the rate of antioxidants recovered was proportional to the extraction temperature. Finally, the developed model allowed to determine the optimal conditions for phenolic compounds extraction using CSE from grapevine leaves. Using Eq. (1) the following parameters were obtained: 29.4% concentration of ethanol (v/v); 37.5 °C temperature; 31 min time; and 72:1 liquid-to-solid ratio. Using these values the predicted TPC yield was 47 ± 2 mg GAE/g. In order to verify the predictive capacity of the model, the best conditions were applied o obtain a grapevine leaves extract giving an experimental TPC value of 46 ± 2 mg GAE/g, which is significantly in agreement with the model.

### Optimization of MAE conditions

The TPC values provided by the Box–Behnken Design experiments are reported in Table S2. The mathematical model used in the Box–Behnken plan for four factors is a classical second-degree model without effects of interactions, deemed insignificant in the analysis [35]. Using this model the following predictive equation was obtained:

$$\begin{aligned} Y(TPC) = {} & 52.00 - 0.12X_1 + 1.93X_2 - 0.29X_3 - 2.91X_4 \\ & + 1.06X_1X_2 - 7.44X_1X_3 - 5.36X_2X_3 \\ & + 3.85X_1X_4 + 1.57X_2X_4 + 7.33X_3X_4 \\ & - 6.02X_1^2 - 2.86X_2^2 + 2.77X_3^2 - 12.64X_4^2 \end{aligned} \quad (2)$$

[$X_1$ concentration of ethanol (%, v/v); $X_2$ power (W); $X_3$ time (s); and $X_4$ liquid-to-solid ratio (mL/g)].

The analysis of variance of the BBD quadratic model obtained using MAE process is summarized in the Table 2. The $R^2$ and $R^2$-adjusted values obtained by the model are 0.98 and 0.94, respectively. These values are reasonably close to 1.0, which indicates a good fit between the observed and predicted values. The $p$ value for lack of fit is 0.61, which is not significant. Finally, the relatively low RMSE value of 1.8 confirms the accuracy of the mathematical model.

The main parameter is the liquid-to-solid ratio. TPC value is positively correlated to this ratio up to 60:1 and decrease for higher ratio. Similar effect was observed for the extraction of phenolic compounds from grapevine pomace [29]. In

**Table 2** Analysis of variance (ANOVA) of the quadratic model obtained with microwave assisted extraction

| Source | Sum of squares | df[a] | F value | P value > F | |
|---|---|---|---|---|---|
| Model | 1682.34 | 14 | 36.059 | < 0.0001 | Significant |
| $X_1$–ethanol | 0.192 | 1 | 0.058 | 0.814 | |
| $X_2$—power | 44.776 | 1 | 13.436 | 0.003 | |
| $X_3$—time | 1.068 | 1 | 0.320 | 0.582 | |
| $X_4$—ratio | 101.617 | 1 | 30.493 | 0.0001 | |
| $X_1X_2$ | 4.494 | 1 | 1.349 | 0.268 | |
| $X_1X_3$ | 221.563 | 1 | 66.485 | < 0.0001 | |
| $X_2X_3$ | 115.133 | 1 | 34.548 | < 0.0001 | |
| $X_1X_4$ | 59.367 | 1 | 17.814 | 0.001 | |
| $X_2X_4$ | 9.860 | 1 | 2.959 | 0.111 | |
| $X3X_4$ | 215.062 | 1 | 64.535 | < 0.0001 | |
| $X_1^2$ | 193.844 | 1 | 58.168 | < 0.0001 | |
| $X_2^2$ | 43.663 | 1 | 13.102 | < 0.0001 | |
| $X_3^2$ | 41.107 | 1 | 12.335 | 0.003 | |
| $X_4^2$ | 852.947 | 1 | 255.948 | < 0.0001 | |
| Residual | 39.990 | 12 | | | |
| $R^2$ | | | | 0.97 | |
| Adjusted $R^2$ | | | | 0.94 | |
| Lack of fit | 33.090 | 10 | 0.959 | 0.612 | Not significant |
| Pure error | 6.900 | 2 | | | |
| Cor total | 1722.335 | 26 | | | |

[a]*df* degrees of freedom

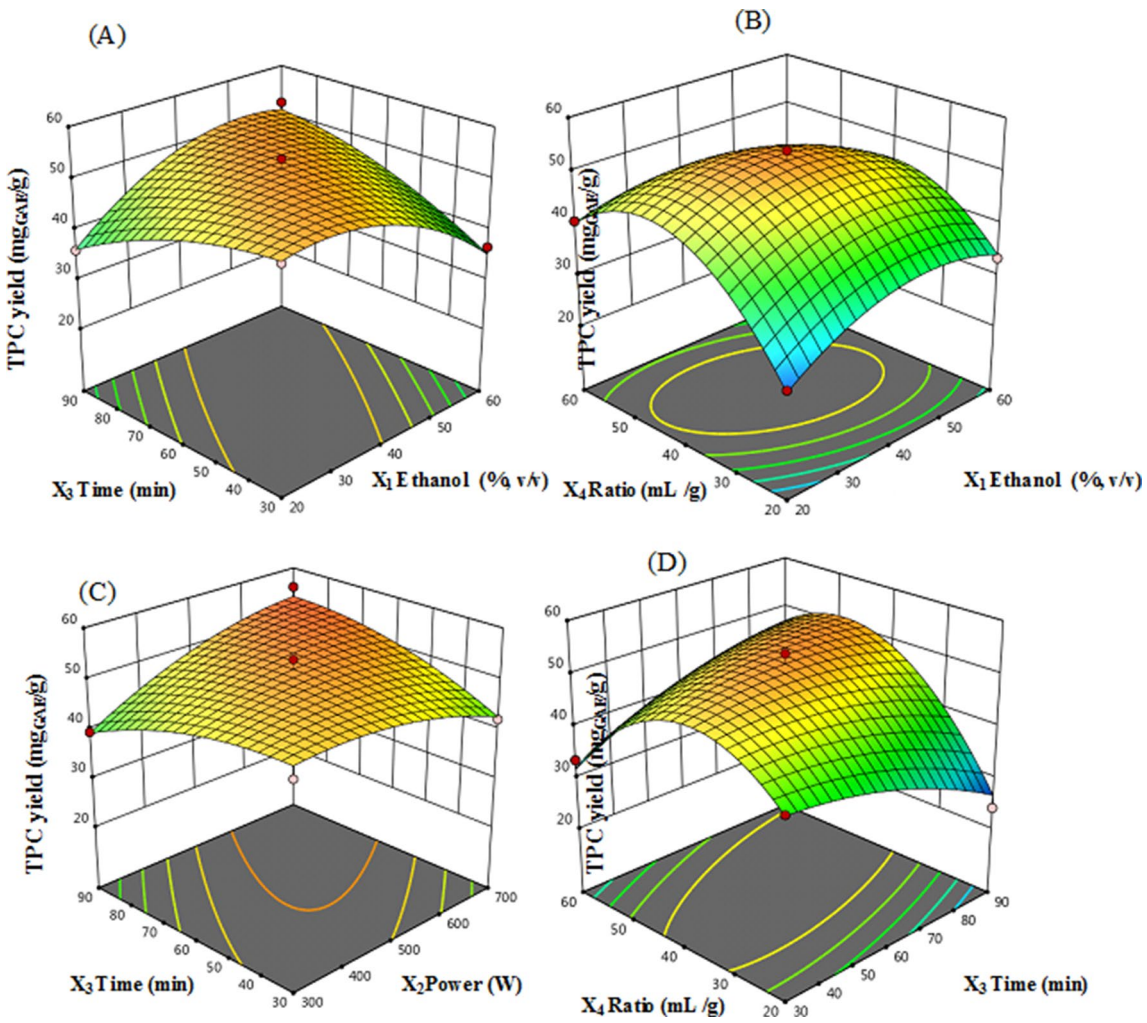



**Fig. 2** Response surface analysis of the effect of microwave assisted extraction on TPC value of grapevine leaves extracts: **a** irradiation time and ethanol concentration, **b** solid-to-liquid ratio and ethanol concentration, **c** irradiation time and microwave power, **d** solid-to-liquid ratio and irradiation time

addition, the microwave power has also a significant impact on the TPC value, as quadratic and interaction effects. Effectively, several works have confirmed the influence of microwave power on plant tissues [27, 36, 37]. The results show that the TPC value is not influenced by the concentration of ethanol but its interaction with time and the liquid-to-solid ratio have an effect on the process. Furthermore, the extraction time does not influence the TPC yield, contrary to its interaction and quadratic terms combined with concentration of ethanol, microwave power, and the solid-to-liquid ratio.

The response surfaces of MAE on TPC value were presented in Fig. 2. The response surface plots confirm the significant impact of liquid-to-solid ratio on TPC value as illustrated by Fig. 2b, d. The TPC value is positively correlated to the increase of the liquid-to-solid ratio, reaching to saturated value for 60 s extraction time at 500 W microwave power (Fig. 2b), and 40% concentration of ethanol concentration at 500 W (Fig. 2d), then decreases until 80:1 (mL/g). Consequently, the ratios 20:1 and 60:1 (mL/g) are best suited for a better recovery TPC. A high concentration of ethanol could increase the diffusion rate, and the solubility of the phenolic compounds, which is governed by the polarity of the solvent [38]. TPC value increases with the enhancement of microwave power until 500 W for 90 s extraction time and then stabilized up to 900 W (Fig. 2c). This effect could be due to the microwave energy impact on plant material by ionic conduction and dipole rotation [39]. The microwaves induce volumetric power which generates molecular agitation and heating. For higher microwave power (> 400 W) an alteration of some phenolics could occur as previously reported by Li, Li, Lin, Zhang, Zhao and Li [40]. To preserve the structure of the compounds, moderate microwave power should be applied for a short time.

Concentration of ethanol and extraction time conjugated parameters have significant positive effect on TPC value (Table 2 and Fig. 2a). The TPC value increases with concentration of ethanol gradient from 20 to 60% and extraction time 30 to 90 s, reaching to a maximum for 40% concentration of ethanol. For the higher concentrations of ethanol and extraction time, negative effect is observed. Water-alcohol mixtures have been shown to be more effective than pure organic solvents for phenolic compounds extraction [41, 42]. Water/alcohol mixtures with low concentration of ethanol improve the entrance to cells, whereas high concentration induces protein denaturation, precluding phenolic compounds solubility [43]. For long times of extraction, compound oxidation could occur. In addition, due to their dielectric properties, solvents such as water or alcohol can heat up impacting the structures of the thermolabile compounds [44].

To conclude, using the Eq. (2), the ideal conditions for phenolic extraction by the MAE from grape leaves are: 474 W microwave power for a duration of 47 s with 40:1 (mL:g) liquid-to-solid ratio and 34% concentration of ethanol. The TPC value predicted by the model is $52 \pm 3$ mg GAE/g. This value is very close to the experimental one $54 \pm 2$ mg GAE/g.

## Comparison between MAE and CSE

The TPC, TFC and antioxidant activities (DPPH, ORAC, PFRAP and ICA) from grapevine leaves (Le Tizourine Bou Afraraet variety) extracts by applying MAE and CSE procedures are provided in Table 3.

The TPC value obtained by CSE (45.6 mg GAE/g) is significantly lower than MAE one (53.6 mg GAE/g). The same tendency is noted for TFC values (18.5 and 32.4 mg for CSE and MAE, respectively). Concerning antioxidant activities, MAE increases significantly these capacities except for iron chelating (ICA). With regard to ICA, the CSE revealed a higher activity than MAE (9.2 and 8.2 mg EDTA/g, respectively).These results indicate that MAE is more efficient to extract bioactive phenolic compounds form grapevine leaves than classical solvent extraction. Similar findings were obtained concerning phenolic compounds form different plant sources [40, 45]: triaterpene acids from olive skins [46], and pectin from spineless cactus [47]. Microwave energy can break cell structure, promoting the release of phenolic compounds [48, 49]. Time of exposure to microwaves is an important parameter to be considered. Due to heat effect, the time of extraction is shorter in MAE than in CSE process (Tables S1 and S2), avoiding compound degradation [44]. The lower antioxidant properties of CSE extract may be a result of the longer time of extraction using this process.

MAE is an efficient process for extracting natural compounds from plant sources, reducing waste, avoiding water and solvent consumption, with special care for thermolabile constituents [50]. These recovered bioactive compounds could be used as food additive to fortify or supplement certain food inputs [51].

**Table 3** Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity (DPPH, ORAC, PFRAP and ICA) from Le Tizourine Bou Afraraet leaves extracts obtained under the best conditions with conventional solvent extraction (CSE) and microwave-assisted extraction (MAE)

| Extraction method | TPC (mg GAE/g) | TFC (mg QE/g) | DPPH (mg of TE/g) | ORAC (mg of TE/g) | PFRAP (mg EAA/g) | ICA (mg EDTA/g) |
|---|---|---|---|---|---|---|
| CSE | $45.6 \pm 1.3^{b}$ | $18.5 \pm 0.1^{b}$ | $195.7 \pm 9.4^{b}$ | $1463 \pm 169^{b}$ | $248 \pm 11^{b}$ | $9.2 \pm 0.8^{b}$ |
| MAE | $53.6 \pm 2.1^{a}$ | $32.4 \pm 0.8^{a}$ | $267.6 \pm 8.9^{a}$ | $2375 \pm 253^{a}$ | $279 \pm 11^{a}$ | $8.2 \pm 0.5^{a}$ |

Values are the means of three determinations ± standard deviation. Same letters in the same column refer to means not statistically different ($p > 0.05$) according to ANOVA and Tukey's tests

*TE* trolox equivalent, *GAE* gallic acid equivalent, *EAA* ascrobic acid equivalent, *EDTA* Ethylene Diamine Tetraacetic Acid

**Table 4** Comparison between the two varieties of grapevine leaves Le Tizourine Bou Afraraet and Ahmar-Bou-Amar extracted by MAE in term of Total phenolic content (TPC), Total Flavonoid Content (TFC) and antioxidant activity (DPPH, ORAC, PFRAP and ICA)

| Variety | TPC (mg GAE/g) | TFC (mg QE/g) | DPPH (mg of TE/g) | ORAC (mg of TE/g) | PFRAP (mg EAA/g) | ICA (mg EDTA/g) |
|---|---|---|---|---|---|---|
| Ahmar Bou Amar | $63.9 \pm 1.1^{a}$ | $42.2 \pm 1.7^{a}$ | $264 \pm 5^{a}$ | $2401 \pm 206^{a}$ | $310 \pm 6^{a}$ | $9.2 \pm 0.74^{b}$ |
| Le Tizourine Bou Afraraet | $53.6 \pm 2.1^{b}$ | $32.4 \pm 0.8^{b}$ | $268 \pm 9^{a}$ | $2375 \pm 254^{a}$ | $280 \pm 11^{b}$ | $12.6 \pm 1.8^{a}$ |

Values are the means of three determinations ± standard deviation. Same letters in the same column refer to means not statistically different ($p > 0.05$) according to ANOVA and Tukey's tests

*TE* trolox equivalent, *EAA* ascrobic acid equivalent, *GAE* gallic acid equivalent, *EDTA* Ethylene Diamine Tetraacetic Acid

## Comparison between two varieties of grapevine leaves extracted by MAE

As MAE proved its ability to extract phenolic compounds from grapevine leaves, a microwave extraction was carried out on leaves of another native variety of grapevine, are done, named Ahmar-Bou-Amar grown exclusively in the region of Kabylie in Algeria. MAE was carried out under the predicted optimal conditions obtained for Le Tizourine Bou Afraraet cultivar. A comparison between the leaves extracts obtained from the two varieties of red and white grapevine is made in term of TPC, TFC, and antioxidant properties (DPPH, ORAC, PFRAP and ICA). Results are reported in Table 4. Ahmar-Bou-Amar grapevine leaves extract presents highest TPC and TFC values (63.9 and 42.2 mg/g, respectively).In term of TPC, these findings are similar to that of [52] using classical solvent extraction. TPC values are ranged from 27 to 76 mg GAE/g in grapevine leaves harvested from different regions from Serbia. Concerning TFC, the values are close to that reported by [53] using classical methods. For antioxidant properties, both varieties extracts exhibited comparable scavenging activities in DPPH and ORAC assays. These values are in agreement to that of grapevine leaves extract reported in the literature [54, 55]. PFRAP assays reveal that Ahmar-Bou-Amar variety presents higher antioxidant activities than Le Tizourine Bou Afraraet cultivar. In contrast for ICA assays, Le Tizourine Bou Afraraet exhibits the higher activity. These results indicate that grapevine leaves extracts may be sources of bioactive phenolic compounds. The discrepancy between the results presented in the present study and literature could be due to extraction procedure, sample origin, and period of collection [56, 57].

## Phenolic content

Ahmar-Bou-Amar and Le Tizourine Bou Afraraet grape vine leaves extracts provided under optimized MAE conditions were assayed by HPLC–DAD-IT-MS to determine their phenolic contents for both varieties. Several authors have underlined the importance and relevance of agronomic and environmental factors that affect phenolic compounds from plant material and their bioactivities [4, 58]. Sixteen phenolic compounds are identified and quantified including the following compounds: two phenolic acids (gallic and caftaric acids), three flavanols (catechin, epicatechin and procyanidin B1), seven flavonols (quercetin, quercetin-3-glucoside, quercetin-3-glucoronide, quercetin-3-rutinoside, kaempferol, kaempferol-3-glucoside and kaempferol-3-rutinoside), and four stilbenes (*trans*- and *cis*-resveratrols, *trans*- and *cis*-piceids). Results are presented in Table 5.

Flavonols are the main constituents in both varieties. The main compound identified is quercetin-3-glucuronide in both varieties (34.9 and 19.1 mg/g for Ahmar-Bou-Amar and Le Tizourine Bou Afraraet, respectively), followed by quercetin-3-glucoside (12.8 and 9.83 mg/g for Ahmar-Bou-Amar and Le Tizourine Bou Afraraet, respectively). Kaempferol-3-rutinoside, kaempferol-3-glucoside, and quercetin-3-rutinoside are present in the two varieties with intermediate values. Quercetin and kaempferol present the lowest contents (0.98 and 0.12 mg/g for Ahmar-Bou-Amar and 0.84 and 0.13 mg/g for Le Tizourine Bou Afraraet, respectively). Phenolic acids are represented by two constituents: gallic and caftaric acids. The second one is the main constituent of this class of phenolics in both cultivars (1.86 and 1.66 mg/g for Ahmar-Bou-Amar and Le Tizourine Bou Afraraet, respectively). Flavanols are represented by three compounds. The procyanidin B1 is the main quantified flavanol (0.69 and 0.50 mg/g for Ahmar-Bou-Amar and Le Tizourine Bou Afraraet, respectively), in addition minor amounts of epicatechin and catechin are collected in both varieties. Finally two stilbenes are observed, resveratrol and its glucoside (piceid). Piceid content is higher in both varieties in comparison to resveratrol.

The phenolic composition of grapevine leaves has been previously investigated underlying several compounds [13, 53, 59–61]. The results presented in this work are in agreement with these previous studies. Having knowledge of the composition of grapevine leaves extracts could inform us about their antioxidant properties. According to the current

**Table 5** Content of phenolic compounds (mg/g of extract) in red vine leaves cv. Ahmar-Bou-Amar and white vine leaves cv. Le Tizourine Bou Afraraet extracted by Microwave- Extracted Method (MAE)

| Compound | Ahmar-Bou-Amar | Le Tizourine Bou Afraraet |
|---|---|---|
| Gallic acid | $0.003 \pm 0.00^{a}$ | $0.005 \pm 0.00^{b}$ |
| Cafratic acid | $1.86 \pm 0.40^{a}$ | $1.66 \pm 2.72^{a}$ |
| Epicatechin | $0.06 \pm 0.01^{a}$ | $0.06 \pm 0.01^{a}$ |
| Catechin B1 | $0.69 \pm 0.07^{a}$ | $0.5 \pm 0.10^{b}$ |
| Catechin | $0.03 \pm 0.00^{a}$ | $0.02 \pm 0.00^{b}$ |
| Quercetin-3-glucoside | $12.8 \pm 0.43^{a}$ | $9.83 \pm 1.51^{b}$ |
| Quercetine | $0.98 \pm 0.05^{a}$ | $0.84 \pm 0.17^{a}$ |
| Quercetin glucoronide | $34.88 \pm 5.33^{a}$ | $19.10 \pm 4.94^{b}$ |
| Quercetin-3-rutinoside | $2.79 \pm 0.9^{a}$ | $2.43 \pm 0.76^{a}$ |
| Kaempferol | $0.12 \pm 0.00^{b}$ | $0.13 \pm 0.03^{a}$ |
| Kaempferol-3-glucoside | $2.47 \pm 0.71^{a}$ | $2.83 \pm 4.05^{a}$ |
| Kaempferol-3-rutinoside | $3.05 \pm 0.28^{b}$ | $3.44 \pm 0.52^{a}$ |
| *Trans*-Piceid | $0.13 \pm 0.00^{a}$ | $0.06 \pm 0.00^{b}$ |
| *Cis*-Piceid | $0.26 \pm 0.012^{a}$ | $0.14 \pm 0.01^{b}$ |
| *Trans*-Resveratrol | $0.04 \pm 0.00^{a}$ | $0.01 \pm 0.00^{b}$ |
| *Cis*-Resveratrol | $0.01 \pm 0.00^{b}$ | $0.004 \pm 0.00^{a}$ |

Values are the means of three determinations ± standard deviation

Same letters in the same column refer to means not statistically different ($p > 0.05$) according to ANOVA and Tukey's tests

findings, the leaves of Ahmar-Bou-Amar appear to be richer in polyphenols than that of Le Tizourine Bou Afraraet. This result is in agreement with the antioxidant activities observed in the present work.

## Conclusion

In this present work, the extraction of phenolic compounds by microwave and conventional methods were optimized from *Vitis vinifera* L. cv. Le Tizourine Bou Afraraet leaves. This is the first time that such optimization on grapevine leaves was performed by applying the Box–Behnken experimental design. The extraction parameters and their interactions were investigated to optimize the extraction process. The MAE process provided the highest phenolic content and antioxidant activities, with shorter working time and lower solvent consumption. Furthermore, the phenolic constituents from leaves of another Algerian variety, Ahmar-Bou-Amar, were extracted by the optimized MAE strategy. Results showed that leaves of both considered cultivars were rich in phenolic compounds. These by-products of viticulture may constitute a source of attractive phytochemical constituents for the food industry and/or pharmaceutical applications.

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### Compliance with ethical standards

**Conflict on interest** The authors declare no competing financial interest.

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