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**ARTICLE** *in* FOOD CHEMISTRY · JANUARY 2015

Impact Factor: 3.39 · DOI: 10.1016/j.foodchem.2014.06.066



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Analytical Methods

# Optimization of microwave-assisted extraction of polyphenols from *Myrtus communis* L. leaves

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ARTICLE INFO

*Article history:*
Received 25 August 2013
Received in revised form 7 June 2014
Accepted 15 June 2014
Available online 23 June 2014

*Keywords:*
Myrtus leaves
Microwave extraction
Ultrasound extraction
Phenolic compounds
Antioxidant activity
Response surface method

ABSTRACT

Phytochemicals, such as phenolic compounds, are of great interest due to their health-benefitting antioxidant properties and possible protection against inflammation, cardiovascular diseases and certain types of cancer. Maximum retention of these phytochemicals during extraction requires optimised process parameter conditions. A microwave-assisted extraction (MAE) method was investigated for extraction of total phenolics from *Myrtus communis* leaves. The total phenolic capacity (TPC) of leaf extracts at optimised MAE conditions was compared with ultrasound-assisted extraction (UAE) and conventional solvent extraction (CSE). The influence of extraction parameters including ethanol concentration, microwave power, irradiation time and solvent-to-solid ratio on the extraction of TPC was modeled by using a second-order regression equation. The optimal MAE conditions were 42% ethanol concentration, 500 W microwave power, 62 s irradiation time and 32 mL/g solvent to material ratio. Ethanol concentration and liquid-to-solid ratio were the significant parameters for the extraction process ($p < 0.01$). Under the MAE optimised conditions, the recovery of TPC was 162.49 ± 16.95 mg gallic acid equivalent/g dry weight (DW), approximating the predicted content (166.13 mg GAE/g DW). When bioactive phytochemicals extracted from Myrtus leaves using MAE compared with UAE and CSE, it was also observed that tannins (32.65 ± 0.01 mg/g), total flavonoids (5.02 ± 0.05 mg QE/g) and antioxidant activities (38.20 ± 1.08 μg GAE/mL) in MAE extracts were higher than the other two extracts. These findings further illustrate that extraction of bioactive phytochemicals from plant materials using MAE method consumes less extraction solvent and saves time.



## 1. Introduction

Phytochemicals such as phenolic compounds from plants and vegetables are known to have several health-benefitting properties, including reducing the risks of certain types of cancer, cardiovascular, heart and neurodegenerative diseases. Although there are still some unanswered questions about the effects of polyphenols on human diseases, the health-promoting potential of these foods may be attributed to the phytochemicals present in the roots, barks, stems, leaves, fruits, and flowers of some plants (Song, Li, Liu, & Zhang, 2011; Yang et al., 2010). *Myrtus communis* is one of the important aromatic and medicinal species from the Myrtaceae family (Wannes, Mhamdi, Sriti, & Marzouk, 2010), in Myrtus genus and comprises of about 50 species native to the Mediterranean basin. *M. communis* is a typically Mediterranean sub-shrub (high: 1–3 m) with white flowers (blossoming time: June to July) growing wild in Corsica (altitude: 0–600 m) (Barboni et al., 2010), widely used in herbal medicine in Mediterranean countries, and recently determined to contain very high amounts of hydrolysable tannins and flavonoid glycosides (Tattini et al., 2006). Extraction of bioactive compounds from Myrtle leaves has been investigated in the last decades focusing mainly on conventional solvent extraction. Various efficient and advanced extraction techniques developed for extracting phenolic compounds from herbal medicine, include pressurized liquid extractor (PLE) (Luthria, 2008), microwave-assisted extraction (MAE) (Spigno & De Faveri, 2009), ultrasound-assisted extraction (Jerman, Trebše, & Mozetič Vodopivec, 2010), soxhlet extraction and heat reflux extraction (HRE) (Jun, Deji, Ye, & Rui, 2011), and supercritical fluid extraction (Camel, 2000). Among these, MAE is a relatively new method by which microwave energy is used to heat polar solvents in contact with solid samples and to partition compounds of interest between the sample and the solvent, reducing both extraction time and solvent consumption (Pérez-Serradilla & Luque de Castro, 2011). It also produces higher extraction rates and better results with lower costs (Gallo,

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http://dx.doi.org/10.1016/j.foodchem.2014.06.066
0308-8146/

Ferracane, Graziani, Ritieni, & Fogliano, 2010). However, due to the many factors that influence MAE, optimization of the extraction process parameters is required to retain the maximum amount of phenolic compounds (Spigno & De Faveri, 2009).

In the present study, (i) a response surface method was examined for optimization of MAE process parameters (ethanol concentration, irradiation power, extraction time, and liquid-to-solid ratio) to obtain maximum yield of total phenolics; (ii) determined tannins, flavonoids and antioxidant activity of MAE extracts and compared the quantities using USE and CSE methods.

## 2. Materials and methods

### 2.1. Plant materials

The leaves of *M. communis* were collected from Oued ghir, Bejaia province of Algeria (North East of Algeria-Bejaia; latitude 36.717°; longitude 4.967°, altitude 99 m). Collected leaves were dried in a forced-air oven at 40 °C to constant weight, and then ground using an electric grinder (IKA model-A11, Staufen, Baden-Württemberg, Germany). The ground powder was passed through a standard 125 μm sieve and was collected and stored at 4 °C in airtight bags until further use.

### 2.2. Reagents

Sodium carbonate ($Na_2CO_3$), Folin–Ciocalteu's phenol reagent, disodium hydrogen phosphate ($Na_2HPO_4$), hydrochloric acid (HCl) and chloride aluminium ($AlCl_3$, $6H_2O$) were purchased from Prolabo (Loire, France). 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), polyvinyl polypyrrolidone (PVPP), potassium persulfate and $FeSO_4$ were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Gallic acid was purchased from Biochem-chemopharma (Loire, France). All solvents used were of analytical grade and purchased from Prolabo (Loire, France). Standards for HPLC such as gallic acid, ferulic acid, caffeic acid, p-coumaric acid, chlorogenic acid, rutin, quercetin, catechin and epigalocatechin were purchased from (Fisher scientific, Fair Lawn, NJ, USA). All the solvents used for HPLC analysis were HPLC grade.

### 2.3. Extraction and quantification of total phenolic content (TPC)

#### 2.3.1. Microwave-assisted extraction

Phenolic compounds from powders of Myrtus leaves were extracted using a domestic microwave oven system (2450 MHz, Samsung Model NN-S674MF, Kuala Lumpur, Malaysia). The apparatus was equipped with a digital control system for irradiation time and microwave power (the latter was linearly adjustable from 200 to 1000 W). The oven was modified in order to condense the vapours generated during extraction in to the sample. Different concentrations of ethanol in water were used as a safe and efficient solvent for the extraction of phenolic compounds, (Li et al., 2012).

One gram of Myrtus powder was stirred in to aqueous ethanol by stirring in preparation for extraction using the MAE system. The MAE extraction parameters were microwave power (400–600 W), extraction time (30–90 s), liquid-to-solid ratio (20–40 mL/g) and ethanol proportion (20–100%). Table 1 provides the experimental conditions, where the influence of each parameter was investigated in single-factor experiments. Each trial was carried out in triplicate. After MAE treatment, the extract was filtered through a Whatman No. 1 filter paper lined Büchner funnel and the supernatant was collected in a volumetric flask. The extract was stored at 4 °C until further use.

**Table 1**
Experimental design with the observed responses of total phenolic compounds (TPC) yield from *M. communis* leaves using microwave-assisted extraction (MAE).

| Run | $X_1$ Ethanol concentration (% v/v) | $X_2$ Microwave power (W) | $X_3$ Irradiation time (s) | $X_4$ Solvent-to-solid ratio (mL/g) | Recovery of TPC (mg GAE/g) |
|---|---|---|---|---|---|
| 1 | 20 | 500 | 60 | 20 | 119.50 |
| 2 | 40 | 500 | 90 | 40 | 150.72 |
| 3 | 20 | 500 | 30 | 30 | 156.77 |
| 4 | 40 | 400 | 90 | 30 | 146.53 |
| 5 | 40 | 400 | 30 | 30 | 173.49 |
| 6 | 60 | 600 | 60 | 30 | 157.53 |
| 7 | 20 | 500 | 60 | 40 | 152.40 |
| 8 | 20 | 400 | 60 | 30 | 145.47 |
| 9 | 60 | 400 | 60 | 30 | 149.69 |
| 10 | 60 | 500 | 90 | 30 | 159.18 |
| 11 | 40 | 400 | 60 | 20 | 138.09 |
| 12 | 40 | 500 | 30 | 20 | 143.21 |
| 13 | 60 | 500 | 60 | 20 | 147.43 |
| 14 | 20 | 600 | 60 | 30 | 138.99 |
| 15 | 60 | 500 | 30 | 30 | 153.60 |
| 16 | 40 | 600 | 60 | 20 | 150.44 |
| 17 | 40 | 600 | 30 | 30 | 144.79 |
| 18 | 40 | 500 | 30 | 40 | 161.29 |
| 19 | 40 | 600 | 60 | 40 | 161.74 |
| 20 | 60 | 500 | 60 | 40 | 142.91 |
| 21 | 40 | 600 | 90 | 30 | 171.39 |
| 22 | 40 | 400 | 60 | 40 | 165.51 |
| 23 | 40 | 500 | 90 | 20 | 139.89 |
| 24 | 20 | 500 | 90 | 30 | 145.47 |
| 25 | 40 | 500 | 60 | 30 | 168.97 |
| 26 | 40 | 500 | 60 | 30 | 163.58 |
| 27 | 40 | 500 | 60 | 30 | 161.93 |

GAE: gallic acid equivalent.

#### 2.3.2. Ultrasound assisted extraction (UAE)

An ultrasonic system with working frequency fixed at 20 kHz (SONICS Vibra cell, VCX 130 PB, Stepped microtips and probes, No. 630-0422, Newtown, CT, USA) was used for extraction of phenolic compounds from the Myrtus leaves. Briefly, 1 g of powder was mixed with extraction solvent in a 250 mL amber glass bottle and the suspension was exposed to acoustic waves for 15 min. The temperature (27 ± 2 °C) was controlled by continuously circulating cold water using an external water bath. After the UAE treatment, the supernatant was recovered and analysed as reported for MAE in Section 2.3.1.

#### 2.3.3. Conventional solvent extraction (CSE)

Phenolic compounds in Myrtus leaves were extracted using a conventional solvent extraction method following the procedures of Spigno, Tramelli, and De Faveri (2007). Briefly, 1 g of powder was mixed with 50 mL of 50% ethanol (v/v) in a conical flask and the mixture was kept in a thermostatic water bath (model. WNB22, Memmert, Frankfurt, Germany) at 60 °C, with shaking at a speed of 110 strokes per minute for 2 h. After the CSE treatment, the supernatant was recovered and analysed as mentioned for MAE in Section 2.3.1.

#### 2.3.4. Determination of total phenolic content (TPC)

The total phenolic content in the extracts was determined by the Folin–Ciocalteu method (Jaramillo-Flores et al., 2003). Briefly, 100 μL of supernatant was mixed with 750 μL of a 10-fold diluted Folin–Ciocalteau reagent. The solutions were mixed thoroughly and incubated at room temperature (27 °C) for 5 min. After incubation, 750 μL of 7.5% sodium carbonate ($Na_2CO_3$) solution was added and again incubated at 25 °C for 90 min. The absorbance of the reaction mixtures were measured at 725 nm using a UV–Vis spectrophotometer (Model: SpectroScan 50, Nicosia, Cyprus).

The absorbance of the extract was compared with a gallic acid standard curve for estimating concentration of TPC in the sample. The TPC was expressed as mg of gallic acid equivalents (GAE) per gram of powder on dry weight (DW) basis.

*2.3.5. Determination of flavonoids contents*

The flavonoid content of the extracts was estimated by the $AlCl_3$ method (Quettier-Deleu et al., 2000). Briefly, 1 mL of ethanolic extract solution was added to 1 mL of 2% methanolic $AlCl_3{\cdot}6H_2O$. The absorbance was measured at 415 nm after 10 min of equilibrium and was then compared to a quercitin standard curve for concentration of total flavonoids in the samples. The results were expressed in mg quercitin/g of powder on dry weight basis (mg QE/g DW).

*2.3.6. Determination of tannins*

Condensed tannins in the extracts were determined according to the polyvinyl polypyrrolidone (PVPP) method following the procedures of Makkar, Blümmel, and Becker (1995) and based on the ability of PVPP to bind tannins. Briefly, 0.2 g of PVPP was added to 5 mL of extract and mixed with 15 mL of acidified water (pH, 3). After centrifugation of the mixture at 4600*g* (~6000 rpm) for 10 min, the precipitate was rinsed with 10 mL of distilled water. 20 mL of iron reagent (150 mg/L of iron(II) sulfate in a solution of *n*-BuOH–conc. HCl (50:50, v/v)) were mixed with the precipitate (PVPP-tannins) in glass tubes. The tube was hermetically sealed, shaken and the reaction developed within 30 min in a boiling water bath. The solution was cooled and the absorbance measured at 550 nm. The value was corrected by the blank (a sample prepared in the same way but left for 30 min in the dark without heating) and multiplied by 0.273 (a conversion factor calculated) to give the mg/L of tannins. The tannin concentration is calculated by the following equation Eq. (1):

$$C\left(\frac{\text{mg}}{\text{L}}\right) = 273(D_1 - D_0) \tag{1}$$

where $C$ = Tannins concentration (mg/L),$D_1$ = Optical density of sample at $t_{30\text{min}}$,$D_0$ = Optical density of control at $t_{30\text{min}}$.

*2.3.7. Determination of antioxidant activity*

*2.3.7.1. Scavenging activity against the ABTS radical.* The antioxidant activity of Myrtus leaves was assessed by ABTS assay (Re et al., 1999), which is based on the ability of antioxidants to interact with the ABTS radical, decreasing its absorbance at 734 nm. Briefly, a radical solution (7 mM ABTS and 2.45 mM potassium persulfate) was prepared in ethanol and left to stand in the dark at room temperature (27 °C) for 12–16 h before using in the assay. This solution was then diluted with ethanol to get an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. For the antioxidant activity analysis, 2 mL of the diluted radical solution were mixed with 20 μL of the extracted supernatant at different concentrations and the absorbance was read at 734 nm against ethanol. Antioxidant activity (AOX) was calculated as the percent inhibition of absorbance at 734 nm (Eq. (2)):

$$\text{AOX} = \%\ \text{inhibition} = \frac{A_{\text{blank}(t=6)} - A_{\text{sample}(t=6)}}{A_{\text{ABTS}(t=0)}} \times 100 \tag{2}$$

where $A_{\text{blank}}$ is the absorbance value of the blank (2 mL of ABTS solution plus 20 μL of the solvent in which the extract has been dissolved); $A_{\text{sample}}$ is the absorbance of the sample extract; $t$ is the time (in min) at which absorbance was read. Sample extracts were always diluted to a total phenolic concentration in the range 10–100 $\mu g_{GAE}/g_{dw}$.

*2.3.7.2. Scavenging activity against the DPPH radical.* The free radical scavenging ability of the extract was measured using a colorimetric method where change in the purple colour solution of DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical was measured on a plate reader (Omega FLUOstar, BMG Labtech, Cary, NC, USA). 300 μL of DPPH· solution in methanol (70 μM) was mixed with 10 μL of extract and the mixture was incubated for 20 min at 37 °C. The decrease in absorbance reading of the mixture was measured at 515 nm. The antioxidant capacity of the extract was expressed as a percentage of inhibition of DPPH radical (% inhibition of DPPH radical) calculated according to the following equation (Eq. (3)):

$$\%\text{inhibition} = \text{AOX} = \frac{A_{\text{blank}_{t=20}} - A_{\text{sample}_{t=20}}}{A_{\text{DPPH}_{t=0}}} \times 100 \tag{3}$$

where, $A_{\text{blank}}$ is the absorbance value of the blank (300 μL of DPPH solution plus 10 μL of the solvent in which the extract has been dissolved); $A_{\text{sample}}$, is the absorbance of the sample extract; $t$ is the time (min) at which absorbance was read and $A_{\text{DPPH}}$ is absorbance of the control at time = 0 min. The effective concentration of sample required to scavenge DPPH radical by 50% ($IC_{50}$ value) is obtained by linear regression analysis of dose–response curve plotting between % inhibition and concentrations.

*2.3.7.3. Oxygen radical absorbance capacity (ORAC) assay.* The antioxidant capacity of the extract was also performed using an oxygen radical absorbance capacity (ORAC) assay using a fluorescence plate reader following the procedures of Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002) with modifications. The plate reader was equipped with an incubator and two injection pumps. Briefly, sample extracts of 20 μL with suitable dilution in phosphate buffer saline solution (75 mM, pH 7.0) were loaded to polystyrene 96-well microplates in triplicate based on a randomised set layout.

The samples were diluted to the proper concentration range for fitting the linearity range of the standard curve. After loading 20 μL of sample, standard and blank, and 200 μL of the fluorescein solution into appointed wells according to the layout, the microplate (sealed with film) was incubated for 15 min in the plate reader at 37 °C and then 20 μL of peroxyl generator AAPH (3.2 μM) was added to initiate the oxidation reaction. The plate reader was programmed to inject and record the fluorescence of fluorescein on every cycle. The kinetic reading decrease of Fluorescein intensity (%) was recorded for 60 cycles with 40 s per cycle setting during 40 min at 37 °C. Absorbance readings of the plate were taken every cycle using an excitation wavelength of 485 nm and an emission wavelength of 535 nm until all fluorescence readings declined to less than 25% of the initial values. Four Trolox solutions (6.25, 12.5, 25, 50 μM in phosphate buffer saline solution (75 mM, pH 7.0) were used to establish a standard curve.

The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve (Eq. (4)).

$$\text{AUC} = \left(0.5 + \frac{f_4}{f_3} + \frac{f_5}{f_3} + \frac{f_6}{f_3} + \cdots + \frac{f_i}{f_3}\right) \times \text{CT} \tag{4}$$

where $f_3$ initial fluorescence reading at cycle 3, $f_i$ *is* a fluorescence reading at cycle *i*, and CT is cycle time in minutes. The net AUC of the sample was calculated by subtracting the AUC of the blank. The regression equation between net AUC and Trolox concentrations was determined and ORAC values were expressed as μmol Trolox equivalents per gram of sample (μmol TE/g) using the standard curve established in same condition ($R^2$ = 0.9946).

*2.3.8. HPLC-DAD analysis*

Control and treated sample extracts were fractionated for phenolic acids in four fractions using SEP-PAK columns (Waters Associates, Milford, MA, USA). The columns were activated for neutral

phenolics by sequentially passing 50 mL of ethyl acetate, acidified methanol (0.01% v/v HCl) and acidified water (0.01% v/v HCl). A sample extract volume (fraction 1) of 20 ml was loaded to the column prior to washing the column with 60 ml of acidified distilled water to remove any organic sugars and acids remaining in solution (fraction 2). The absorbed fraction phenolic acids were eluted with 60 ml of ethyl acetate (fraction 3). Anthocyanins plus proanthocyanidins (fraction 4) were eluted with 60 ml of acidified methanol. Finally, all fractions (1, 2, 3 and 4) were collected for the HPLC analysis.

Identification of individual phenolic acids was performed using a High-Performance Liquid Chromatography (Agilent Technologies, Santa Clara, USA) equipped with a Diode array detector and a column C-18 (5 μm, 4.60 mm × 250 mm, USA). All samples were centrifuged at 5000 rpm for 10 min before injection into the column with an injection volume of 20 μL and at a flow rate of 0.5 mL/min. Chromatographic analysis was carried out at 30 °C using simultaneous monitoring of extracts performed at 254, 280, 520, 300 and 700 nm. The mobile phase A was a mixture of 6:94 (v/v) acetic acid in distilled water, whereas mobile phase B consisted of 100% HPLC grade acetonitrile. The solvent gradient in volume ratios was as follows: 0–40 min, 0–25% B; 40–80 min, 25–85% B; 80–90 min, 85–100% B; 90–95 min, 100% B.

Individual phenolic compounds were identified by the retention time and quantified from peak area at 280 nm. Identified phenolic compounds (phenolic acid and flavonoids) were quantified using external standards. The standard response curve was a linear regression fitted to values obtained at each concentration within the range of 12.5–200 μg/mL for phenolic acid (Gallic acid, Ferulic acid, Caffeic acid, p-Coumaric acid and Chlorogenic acid) and 41.5–333 μg/mL for flavonoids (Rutin, Quercetin, Catechin and Epigalocatechin).

#### 2.3.9. Scanning electron microscopy (SEM) analysis

Powder of *M. communis* leaves was observed under SEM (Quanta 200, FEI Company, France) for morphological characterization before and after the extraction processes (Lou et al., 2010). Four samples of the powders (untreated and dried residues of MAE, UAE and CSE samples treated under MAE optimised conditions) were used for SEM analysis. Residues after extraction using MAE, UAE and CSE were dried at 60 °C until constant mass in an air oven for preparing samples for SEM analysis. Dried sample particles were fixed on a specific carbon film support, and their shape and surface characteristics were observed by using gaseous secondary electron detector GSED with environmental mode (ESEM).

## 3. Experimental design and statistical analyses

Influence of the process parameters was investigated using a single-factor-test to determine the preliminary range of the extraction variables including $X_1$ (ethanol concentration), $X_2$ (microwave power), $X_3$ (irradiation time) and $X_4$ (liquid-to-solid ratio). On the basis of the single-factor experimental results (Supplement Table S1), major influence factors and their levels were confirmed. Using a Box-Behnken design (Minitab, version 8.0.7.1, USA), response surface methodology (RSM) was conducted to determine the MAE optimised extraction process variables for maximum recovery of TPC. Table 1 represents the non-coded values of the experimental variables and 27 experimental points. Three replicates (24–27) were used to evaluate the pure error. Experimental design software (DOE) (Minitab, version 8.0.7.1, USA) package was used for the regression analysis of the data to fit a second-order polynomial equation (quadratic model), according to the following general equation (Eq. (5)) which was then used to predict the optimum conditions of the extraction process.

$$Y = B_0 + \sum_{i=1k}^{k} BiXi + \sum_{i=1k}^{k} BiiX^2 + \sum_{i>jk}^{k} BijXiXj + E \quad (5)$$

where $Y$ represents the response function (in this case the TPC yield); $B_0$ is a constant coefficient; $Bi$, $Bii$ and $Bij$ are the coefficients of the linear, quadratic and interactive terms, respectively, and $Xi$ and $Xj$ represent the coded independent variables. According to the analysis of variance, the regression coefficients of individual linear, quadratic and interaction terms were determined. In order to visualise the relationship between the response and experimental levels of each factor and to deduce the optimum conditions, the regression coefficients were used to generate 3-D surface plots and contour plots from the fitted polynomial equation. The factor levels were coded as −1 (low), 0 (central point or middle) and 1 (high), respectively. The variables were coded according to the following equation (Eq. (6)):

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (6)$$

where $x_i$ is the (dimensionless) coded value of the variable $X_i$; $X_0$ is the value of $X$ at the centre point and $\Delta X$ is the step change.

## 4. Result and discussion

### 4.1. Single factor analysis method

#### 4.1.1. Influence of ethanol concentration

The recovery of TPC from the *M. communis* leaves with respect to concentration of ethanol followed a parabolic curve from 20% to 60% (Supplement Fig. S1-A). The yield of TPC increased with increasing amounts of the ethanol concentration in the extraction medium; up to 40%. However, TPC yield began to decline with an increase of ethanol proportion up to 60% in the extraction medium ($p < 0.05$) before increasing again (Supplement Fig. S1-A). Water and low concentration of ethanol can easily get access to cells, but a high concentration of ethanol can cause protein denaturation, preventing the dissolution of polyphenols and then influencing the extraction rate (Yang et al., 2010). Ethanol is a low-polar solvent while water is a strong polar solvent, and they can be blended together in any proportion (Zhang et al., 2007). With the addition of water to ethanol, the polarity of the complex solvent will increase continuously. Phenolic compound molecules are also polar, so the yield of TPC increased with increasing water content according to the "like dissolves like" principle (Zhang, Yang, & Liu, 2008). When the water content of the solvent exceeded approximately 45%, the recovery of TPC was reduced. This may be attributed to the difference in dielectric properties of the solvent towards microwave heating, because it plays an important role in microwave extraction, facilitating heat distribution throughout the sample. In this study, 40% ethanol absorbs microwave energy relatively well and is still a good extraction solvent.

The proportion of ethanol in the extraction solvent was examined at levels between 20% and 60% for the optimization design. The solvent with 40% ethanol content was chosen for the determination of optimal extraction power, time and liquid-to-solid ratio.

#### 4.1.2. Influence of microwave power

The effects of microwave power on the recovery of TPC from Myrtle leaves were investigated at levels ranging from 300 to 900 W with fixed solvent concentration (40% ethanol), irradiation time of 2 min and a liquid-to-solid ratio of 20:1 (mL/g). A significant increase in the recovery of TPC from 134.63 to 152.25 mg GAE/g DW was observed at power levels from 400 to 500 W

(Supplement Fig. S1-B). However, the recovery was reduced significantly beyond 600 W, with the lowest amount observed at 900 W (129.95 mg GAE/g). Reduced recoveries of TPC beyond 600 W could be due to the thermal degradation of the phytochemicals at higher microwave power levels. The heat generated by microwaves, with volumetric heating, in the plant cells may be too strong to breakdown the phytochemicals that were not recovered at higher power levels. These observations revealed that extraction at higher microwave output power levels, which are usually more than 900 W, do not ensure better recovery of phenolic compounds than those extracted at medium power. In a similar study on the effect of extraction of total phenolic acids for mandarin peels using a microwave, Ahmad and Langrish (2012) reported that the yield and amounts of total phenolic acids decreased with increased microwave power (900 W). Based on our observations, moderate microwave power levels of 400, 500 and 600 W were selected as the lower, middle and upper levels, respectively, to apply in RSM optimization.

#### 4.1.3. Influence of irradiation time

Generally, by increasing the extraction time, the quantity of analytes extracted is increased, although there is the risk of the degradation of extracted compounds. In our study, the recovery of TPC was examined at different extraction times (30–210 s) with three other fixed factors: 40% ethanol (v/v), 500 W microwave power level, and 20 mL/g liquid-to-solid ratio. The results indicate that the recovery of TPC increased with the increase of MAE irradiation time in the beginning of extraction with a maximum of 159 mg GAE/g in 60 s followed by a decrease after 90 s (Supplement Fig. S1-C). Our results are in agreement with the findings of Ballard, Mallikarjunan, Zhou, and O'Keefe (2010). The investigators reported better yields of phenolic antioxidants from peanut skins were obtained at 30 s using microwave for extraction. Based on our results, an irradiation time of 60 s was used for further experimentation using MAE for RSM optimization.

#### 4.1.4. Influence of extraction liquid-to-solid ratio

As in other extraction techniques, liquid-to-solid ratio is an important parameter that influences the recovery of phenolic compounds. For example, in an industrial extraction process, it is important to maximise extraction yield, while minimizing consumption of solvent (Spigno & De Faveri, 2009). In the current study, the recovery of TPC from the Myrtus leaves was increased with the increase of liquid/solid ratio during extraction (Supplement Fig. S1-D). It was observed that the recovery of phenolic compounds was maximised at a liquid/solid ratio of 30:1 (mL/g). A ratio of 20–40 (v/w) was further used in the optimization of process parameters during MAE.

### 4.2. Optimization of MAE conditions

#### 4.2.1. Modeling and fitting the model using response surface methodology (RSM)

The experimental design and corresponding response data for the total phenolic content from *M. communis* leaves are presented in Table 1. The regression coefficients of the intercept, linear, quadratic and interaction terms of the model were calculated using the least square technique (Zhang et al., 2013) and are given in Table 2. It was shown that two linear parameters, ethanol concentration ($X_1$) and liquid solvent to solid ratio ($X_4$) and their quadratic parameters were highly significant at the level of $P < 0.01$, whereas all the interaction parameters were insignificant ($P > 0.05$). The interactions $X_1X_4$ and $X_2X_3$ were also significant ($p < 0.01$). Discounting the non-significant parameters ($p > 0.05$), the final predictive equation (Eq. (7)) was obtained as follows:

**Table 2**
Estimated regression coefficients for the quadratic polynomial model and the analysis of variance (ANOVA) for the experimental results of total phenolic contents from *M. communis* leaves.

| Parameter[a] | Estimated coefficients | Standard error | DF[b] | Sum of squares | *F*-value | Prob > *F* |
|---|---|---|---|---|---|---|
| Model | 164.8314 | 2.5597 | 14 | 3515.2629 | 12.7735 | <0.0001 |
| Intercept | | | | | | |
| $B_0$ | 164.8314 | 2.5597 | | 3515.19 | 12.7735 | 0.0056 |
| Linear | | | | | | |
| $X_1$ | 4.3111 | 1.2798 | 1 | 223.03 | 11.3462 | 0.0056 |
| $X_2$ | 0.5084 | 1.2798 | 1 | 3.1025 | 0.1578 | 0.6981 |
| $X_3$ | −1.6655 | 1.2798 | 1 | 33.2808 | 1.6931 | 0.2176 |
| $X_4$ | 8.0007 | 1.2798 | 1 | 768.0868 | 39.0749 | <0.0001 |
| Quadratic | | | | | | |
| $X_1^2$ | −119,645 | 2.2167 | 1 | 763.4926 | 38.8412 | <0.0001 |
| $X_2^2$ | −2.6159 | 2.2167 | 1 | 36.7808 | 1.8711 | 0.1964 |
| $X_3^2$ | −2.2901 | 2.2167 | 1 | 27.9650 | 1.4226 | 0.2560 |
| $X_4^2$ | −11.4404 | 2.2167 | 1 | 698.0128 | 35.5101 | <0.0001 |
| Interaction | | | | | | |
| $X_1X_2$ | 3.5781 | 2.2167 | 1 | 51.2127 | 2.6035 | 0.1325 |
| $X_1X_3$ | 4.2184 | 2.2167 | 1 | 71.1847 | 3.6212 | 0.0813 |
| $X_1X_4$ | −9.3752 | 1.9197 | 1 | 350.08 | 17.8097 | 0.0012 |
| $X_2X_3$ | 13.3898 | 1.9197 | 1 | 717.1516 | 36.4836 | <0.0001 |
| $X_2X_4$ | −4.0301 | 1.9197 | 1 | 64.9678 | 3.3051 | 0.0941 |
| $X_3X_4$ | −1.8142 | 1.9197 | 1 | 13.1553 | 0.6691 | 0.4293 |
| Lack of fit | | | | | | |
| Pure error | | | 10 | 208.7541 | 1.5391 | 0.4571 |
| Residual | | | 2 | 27.1268 | | |
| $R^2$ | | | 12 | 235.8809 | | |
| Adjusted | | | | | 0.942 | |
| $R^2$ | | | | | 0.8637 | |
| C.V. % | 2.9121 | | | | | |
| RMSE | 4.4336 | | | | | |
| Corr. Total | | | 26 | 3751.0777 | | |

[a] Coefficients refer to the general model;
[b] Degree of freedom.

$$Y(TPC) = 164.83 + 4.31X1 + 8X4 - 9.35X1X4 + 13.39X2X3 - 11.96X1^2 11.44X4^2 \quad (7)$$

The analysis of variance (ANOVA) for the experimental results given in Table 2 shows that the model is significant at *F*-value of 12.77. There is only a 0.01% chance that a "Model *F*-Value" this large could occur due to noise. The determination coefficient ($R^2$) was 0.9370, which implied that the sample variations of 93.7% for the MAE efficiency of Myrtle leaves polyphenols were attributed to the independent variables, and only 6.3% of the total variations could not be explained by the model. However, a large value of $R^2$ does not always indicate that the regression model is a sound one (Karazhiyan, Razavi, & Phillips, 2011). In a good statistical model, $R^2$ adj should be comparable to $R^2$. As shown in Table 2, $R^2$ and $R^2$ adj values for the model did not differ greatly. The "Lack of Fit *F*-value" of 1.54 implies that the Lack of Fit is not significant relative to pure error ($p > 0.05$) confirming the validity of the model. The value of coefficient of variation (C.V. %) was 2.91% and "Adequate Precision" ratio of 16.55 suggested that the model was reliable and reproducible agreeing previous reports (Chen, Wang, Zhang, & Huang, 2012). In general, a C.V. higher than 10% indicates that variation in the mean value is high and does not satisfactorily develop an adequate response model (adequate signal for the model) (Karazhiyan et al., 2011). The results indicated that the model could work well for the prediction of TPC extract from Myrtle leaves.

#### 4.2.2. Analysis of response surfaces

To investigate the interactive effects of the independent variables and their mutual interaction on the extraction recovery of phenolic compounds, three dimensional response surface profiles

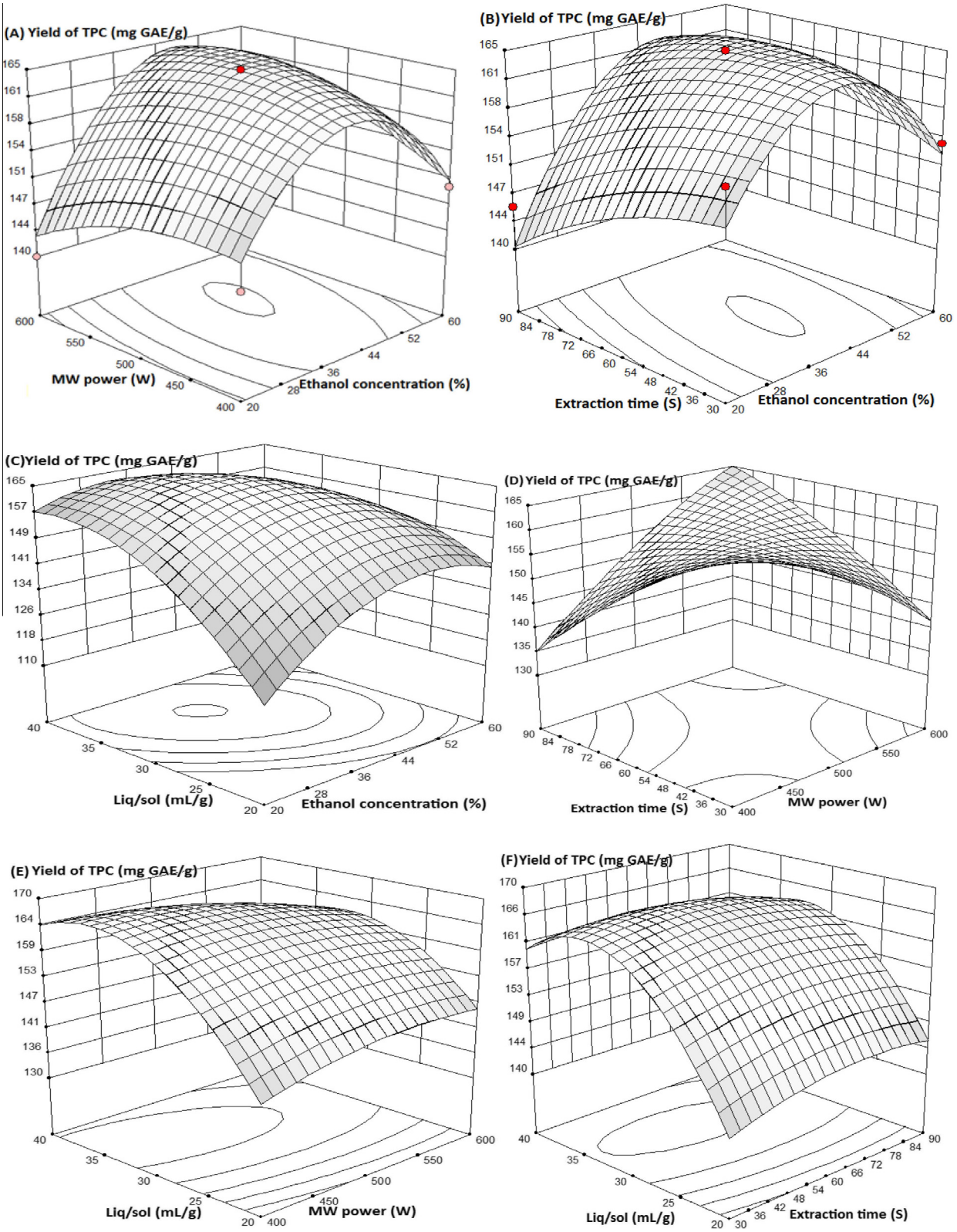


**Fig. 1.** Response surface analysis for the total phenolic yield from *Myrtus communis* peels with microwave assisted extraction with respect to ethanol concentration and microwave power (A); ethanol concentration and extraction/irradiation time (B); ethanol concentration and solvent-to-solid ratio (C); extraction/irradiation time and microwave power (D); microwave power and solvent-to-solid ratio (E); extraction/irradiation time and solvent-to-solid ratio (F).

of multiple non-linear regression models were plotted (Fig. 1A–F). The plots were generated by plotting the response using the *z*-axis against two independent variables (ethanol concentration ($X_1$) and microwave power ($X_2$)) while keeping the other two independent variables (irradiation time ($X_3$) and liquid-to-solid ratio ($X_4$)) at their zero level (Hayat et al., 2009).

Fig. 1A–C depict the interactions between the amount of ethanol concentration and each of the three other factors (MW power,

irradiation time and liquid solvent-to-solid ratio) on the recovery of total phenolic content. The recovery of TPC from Myrtus leaves increased from 145 to 163.80 mg GAE/g DW with the increase of ethanol concentration from 20% to 40% and extraction power from 400 to 500 W and nearly reached a peak at the 40% of ethanol concentration tested. After that, additional ethanol concentration and extraction power caused negative effects. The best point of balance should be sought for the maximum extraction rate of polyphenols between ethanol concentration and extraction power. (Fig. 1A). As shown in Table 3, the recovery of TPC mainly depends on the ethanol concentration as its quadratic and linear effects were highly significant ($p < 0.01$), which result in a curvilinear increase in TPC yield for all the extraction powers and times tested (Fig. 1A and B). Increase in the TPC suggests that the phenolic compounds are more soluble in ethanol/water 45%, confirming the single-factor experiments results. Ethanol could facilitate an increase in the extraction yields and water could enhance swelling of cell material, favourably increasing the contact surface area between plant matrix and solvent, resulting in increase of the extraction yield (Hayat et al., 2009). Fig. 1C shows that the quantity of TPC increased with the increase in ethanol concentration and extraction liquid-to-solid ratio at the beginning followed by a decrease at medium values. The graphs suggested that extracting liquid-to-solid ratio has a quadratic and linear effect ($p < 0.01$) on the yield of TPC (Table 2). The TPC yield could be maximised using a ratio about 30 (mL/g, v/w) over a range of the other operational factors (microwave power and irradiation time). Higher microwave power with longer irradiation time results in a continuous higher temperature in the extraction system. This combination of temperature and time could enhance the solubility of phenolic compounds and decrease the viscosity of extraction solvent, thus accelerating the release and dissolution of these compounds (Fig. 1D). However, high temperatures can also lead to degradation of certain phenolic compounds. The interaction effect of irradiation time ($X_2$) and microwave power ($X_3$) had a significant influence on the acquired ratio of TPC ($p < 0.01$). The linear and quadratic effects of these parameters ($X_2$, $X_3$) were insignificant ($p > 0.05$) while the synergistic effect was highly significant ($p < 0.01$). The recovery of TPC by using MW energy was found to be a function of the interaction effect of extraction power and time.

In fact, in microwave-assisted extraction, microwave power is one of the key variables affecting the release of polyphenols from different matrices by rupturing cell the wall, which also has the ability to modify equilibrium and mass transfer conditions during extraction. Increase in the microwave power accelerated polyphenols extraction. Fig. 1E shows that with an increase of power from 400 to 500 W, the extraction yields of total phenolic compounds increased gradually, followed by a decline with further increase of liquid-to-solid ratio (beyond 30 mL/g). This yielding trend TPC could be attributed to increase in ratio liquid-to-solid that decelerated mass transfer resulting from the lower heating efficiency under microwave conditions and the solubility of polyphenols. In Fig. 1F, when the 3-D response surface plot was developed for the recovery of TPC with varying extraction time and liquid-to-solid ratio, it can be seen that maximum recovery of TPC was achieved with extraction time of 60 s at a liquid-to-solid ratio of 30 (v/w).

#### 4.2.3. Validation and verification of predictive model

The results of experiments that were performed at the optimised extraction conditions using microwave are shown in Table 3. The stationary point giving a maximum MAE efficiency (optimal conditions of MAE) of TPC was obtained using a degree of experiment with the following critical values: 42% ethanol concentration, 515 W microwave power, 62.23 s irradiation time and a liquid-to-solid ratio of 32 mL/g. The appropriateness of the model equation for predicting the optimum response values was tested using the above selected optimal conditions. The predicted extraction yield of TPC was 166.13 mg GAE/g DW that was consistent with the experimental yield of 162.49 mg GAE/g DW. The predicted values were in close agreement with experimental values and were found to be not significantly different ($p > 0.05$) using a paired $t$-test (Hossain et al., 2012). The predicted response values slightly deviated from the experimental data. From preliminary data, the normal probability at residuals indicated no abnormality in the methodology adopted. The strong correlation between the real and predicted results confirmed that the response of regression model was adequate to reflect the expected optimization (Zhang et al., 2013).

### 4.3. Comparison of MAE with other extraction methods

The quantity of TPC, total flavonoids, condensed tannins and antioxidant activity extracted from *M. communis* leaves using MAE, UAE and CSE methods is presented in Table 3. The results indicated that MAE showed a similar extraction capacity for the TPC as compared to USE and CSE ($p > 0.05$). However, extracted tannins using MAE (32.65 ± 0.01 mg/g) were significantly ($p < 0.01$) higher than that of USE and CSE i.e. 23.32 ± 0.01 and 17.18 ± 0.01 mg/g, respectively. In addition, the extracted flavonoids of MAE (5.02 ± 0.05 mg QE/g) were also significantly higher ($p < 0.05$) than that of UAE (3.88 ± 0.45 mg QE/g) and similar ($p > 0.05$) to CSE (4.15 ± 0.75 mg QE/g). The higher extraction yield of total flavonoids, condensed tannins with shorter extraction time could be due to ionic conduction and water dipole rotation effects; the main mechanism of microwave heating. Pressure builds up within the cells of the sample leading to an efficient delivery to plant materials through molecular interaction with the electromagnetic field and a rapid transfer of energy to the extraction solvent and raw plant materials (Chan, Yusoff, Ngoh, & Kung, 2011).

#### 4.3.1. Antioxidant activity

The total antioxidant activity of *M. communis* leaves was determined using ABTS, DPPH radical scavenging and ORAC assay. Comparison of the mean total antioxidant activity of samples is presented in the Table 3. ABTS radical cation decolourisation assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain breaking antioxidants. In this assay, $ABTS^{\cdot+}$ is produced by reacting 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonate] (ABTS) with potassium persulfate ($K_2S_2O_8$). This radical has a relatively stable blue-green colour, which is measured at 734 nm. Antioxidants in the extracts

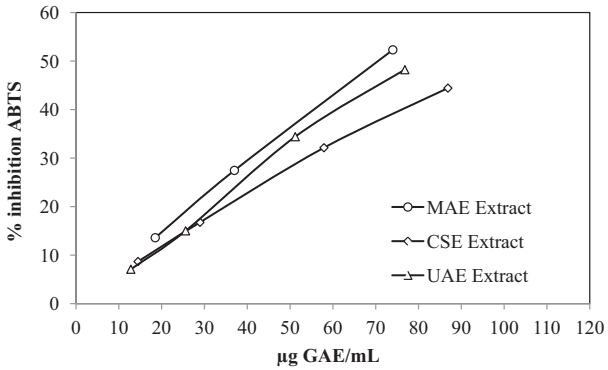


**Fig. 2.** Reducing capabilities of microwave-assisted extracted (MAE) samples compared to ultrasound-assisted extraction (UAE) and conventional-solvent extraction (CSE). Reducing capacity of the extracted was quantified as % inhibition using ABTS radicals.

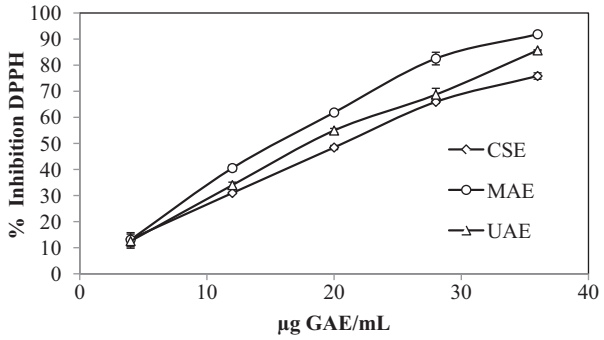


**Fig. 3.** Reducing capabilities of microwave-assisted extracted (MAE) samples compared to ultrasound-assisted extraction (UAE) and conventional-solvent extraction (CSE). Reducing capacity of the extracted was quantified as % inhibition using DPPH radicals.

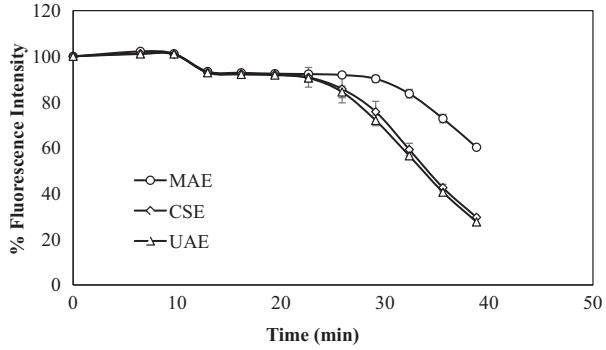


**Fig. 4.** Fluorescence intensity decay of microwave-assisted extracted (MAE) samples compared to ultrasound-assisted extraction (UAE) and conventional-solvent extraction (CSE).This curve was used to determine the antioxidant activity of *Myrtus communis* extracts using ORAC assay.

reduce intensity of this colour to a degree that is proportion to their antioxidant concentration or activity (Pan et al., 2010). Free radical scavenging properties of Myrtus leaves ethanolic extracts using different extraction methods are presented in Table 3. Lower $IC_{50}$ value indicated the greater hydrogen donating ability, thus the higher antioxidant activities (Fig. 2). All ethanolic extracts of Myrtle leaves ($IC_{50}$ = 73.71 ± 1.08 µg/mL for MAE, $IC_{50}$ = 77.96 ± 2.19 µg/mL for UAE, $IC_{50}$ = 96.52 ± 1.93 µg/mL CSE) showed higher scavenging ability on ABTS radicals when compared to the other plant materials reported in literature (Dudonne, Vitrac, Coutiere, Woillez, & Mérillon, 2009). In this study, MAE extracts exhibited the stronger antioxidant activities compared to CSE extracts ($p < 0.01$) and similar than that of the extracts obtained by UAE ($p > 0.05$).

DPPH (a stable, deep purple colour radical) is reduced in the presence of antioxidants decolourizing the solution. Loss of colour results in a decrease in the absorbance intensity, which can be monitored spectrophotometrically at 515 nm, provides the basis for measurement of the antioxidant capacity of the extracts (Fig. 3). Using DPPH radial for the antioxidant capacity of the extracts, MAE showed significantly ($p < 0.01$) lower $IC_{50}$ (16.80 ± 0.29 µg/mL) than UAE (20.61 ± 1.66 µg/mL) and CSE (21.56 ± 0.10 µg/mL) (Table 3).

In the ORAC assay, the decay in the fluorescence intensity was given in Fig. 4. In the assay, extracts using MAE was significantly higher ($p < 0.05$) ORAC value (757.77 ± 31.18 µmol TE/g) than UAE (428.48 ± 28 µmol TE/g) and CAE (459.00 ± 53.49 µmol TE/g) (Table 3). Although it was mentioned earlier that the TPC of extracts obtained using MAE was similar than that found with the UAE and CSE methods, MAE had higher ORAC value than USE and CSE (see Table 3).

Using all the three assays (ABTS, DPPH and ORAC), the antioxidant activities were higher in extracts using MAE compared to CSE and UAE. The higher TPC found with MAE may be explained by breaking down of plant cells in short time with electromagnetic waves after exposure to microwave heating. MAE has noticeably considerable advantages such as shorter extraction time, higher extraction yield and less solvent consumption compared to conventional extraction method (Dahmoune et al., 2013).

*4.3.2. Determination of phenolic compounds by HPLC analysis*

The identification and quantification of individual phenolic compounds in all the fractions of *M. communis* extracts was based on a combination of retention times and calibration curve of external standards using a reverse phase column in HPLC (Fig. 5A–D). The peaks of the phenolic acids were detected at a wavelength of 280 nm. Among the individual phenolic acids in the extracts, galloylquinic acid ($\lambda_{max}$: 274 nm) was eluted at 5.89 min as determined by comparison of spectra and previously reported data (Romani, Campo, & Pinelli, 2012) (Table 4). Gallic acid and epigalocatechin were identified by comparing its retention time and spectral data (gallic acid showing low emission signal) with its authentic standards using HPLC-DAD signal. Our results agreed to previous studies on phenolic compounds present in Italian and Tunisian myrtle leaves (Messaoud, Laabidi, & Boussaid, 2012; Wannes et al., 2010) (Table 4). Romani et al. (2012) reported that gallic acid in Tunisian variety was abundant while Epigalocatechin was abundant in the Italian variety. Peaks of myricitrin and myricetin derivatives such as myricetin-3-O-galactoside, myricetin galloylgalactoside and myricetin-3-O rhamnoside ($\lambda_{max}$: 260 and 357; 266 and 357 nm, respectively) were also detected in the extracts. In addition, a number of other phenolic acids were also detected in the extracts that were not identified.

Effects of MAE and UAE on the individual phenolic acids in different fractions of the samples were compared with CSE samples. Some of the individual phenolic acids were identified by comparing the spectra and elution times of reported literatures under similar conditions. In fraction 1, galloylquinic acid (rt = 5.89) had the higher peak absorbance from MAE compared to UAE and CSE samples (Fig. 5A). Absorbance of another unknown peak was also found higher from MAE and UAE. For fraction 2, galloylquinic acid peak

**Table 3**
Comparison of extraction yield of polyphenols from *M. communis* leaves by microwave assisted (MAE), Ultrasound assisted (UAE), and Conventional solvent extraction (CSE). Results are expressed as means ± standard deviation.

| Extraction method | Extraction time (min) | Ethanol proportion (%) | MW power (w) | Ratio of liquid to solid (mL/g) | Recovery of TPC (mg GAE/g) | Recovery of total flavonoids (mg QE/g) | Recovery of condensed tannins(mg/g) | $IC_{50}$ (µg GAE/mL) | | ORAC value (µmol TE/g) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | ABTS scavenging | DPPH scavenging | |
| MAE | 1.04 | 42 | 500 | 32 | 162.49 ± 16.95$^a$ | 5.02 ± 0.05$^a$ | 32.65 ± 0.01$^a$ | 38.20 ± 1.08$^a$ | 16.80 ± 0.29 $^b$ | 757.77 ± 31.18$^a$ |
| UAE | 15 | 50 | – | 50 | 144.77 ± 30.23$^a$ | 3.88 ± 0.45$^b$ | 23.32 ± 0.01$^b$ | 71.81 ± 2.19$^b$ | 20.61 ± 1.66$^a$ | 428.48 ± 28.00$^b$ |
| CSE | 120 | 50 | – | 50 | 128.00 ± 18.07$^a$ | 4.15 ± 0.75$^a$ | 17.18 ± 0.01$^c$ | 39.85 ± 1.13$^a$ | 21.56 ± 0.10$^a$ | 459.00 ± 53.49$^b$ |

Same letters in the same column refer to means not statistically different ($p > 0.05$).

**Table 4**
Elution times and maximum absorbance of individual phenolic acids present in *M. communis* leaves. Elution times and maximum absorbance of individual phenolic acids were determined using a reverse phase C18 column in HPLC. The mobile phase A was a mixture of 6:94 (v/v) acetic acid in distilled water and mobile phase B consisted of 100% HPLC grade acetonitrile. The solvent gradient in volume ratios was as follows: 0–40 min, 0–25% B; 40–80 min, 25–85% B; 80–90 min, 85–100% B; 90–95 min, 100% B.

| Compounds | $t_r$ (min) | *l* max UV–Vis (nm) | References |
|---|---|---|---|
| (1) Galloylquinic acid | 5.89 | 274 | Romani et al. (2012) |
| (2) Gallic acid | 8.81 | 270 | External standard |
| (3) Gallotannin | 17.52 | 266 | Romani et al. (2012) |
| (4) Myricetin 3-O-galactoside | 33.67 | 260, 357 | Romani et al. (2012) |
| (5) Digalloylquinic | 25.17 | 276 | Romani et al. (2012) |
| (6) Trigalloylquinic HHDD-glucose | 25.94 | 274 | Romani et al. (2012) |
| (7) Myricetin galloylgalactoside | 30.27 | 266, 356 | Romani et al. (2012) |
| (8) Epigalocatechin | 19.69 | 240, 274 | External standard |
| (9) Myricetin 3-O-rhamnoside | 37.77 | 262, 352 | Romani et al. (2012) |
| (10) Quercetin 3-O-rhamnoside | 38.92 | 266, 348 | Romani et al. (2012) |

absorbance almost similar as in CSE, UAE and MAE (Fig. 5B). Gallic acid (peak 2) from MAE had similar peak absorbance as in CSE. However, peak absorbance of gallic acid was lower in UAE samples compared to other two. Similar trend as gallic acid was also observed in UAE samples for same unknown phenolic acids. Most of the phenolic acids identified were observed in fraction 3. All the phenolic acids had higher peak absorbance for MAE samples than UAE and CSE (Fig. 5C). In fraction 4, only few peaks were observed including peak 1 (Fig. 5D). From the peak absorbance it could not be concluded the best method to detect peaks in fraction 4. In overall, the higher peak absorbance of many phenolic acids in MAE samples could be attributed to the easy extraction of the phenolic compounds due to cell damage by microwave with inner penetration.

### *4.3.3. Scanning electron microscopy (SEM)*

The residues of treated Myrtus leaves using MAE, UAE and CSE were examined for structural analyses by scanning electron microscopy and compared with the untreated control. Fig. 6A–D displays the micrographs with structural features of the untreated, CSE, UAE and MAE samples. It was observed that there was complete parenchyma without any significant destruction on cell walls but with slight ruptures on the surfaces of CSE sample compared to untreated one (Fig. 6A–D). After microwave treatment, the surface of the sample was greatly destroyed and the texture was crumbled because of the potential of electromagnetic waves to sudden temperature rise during microwave irradiation and internal pressure increase due to high vapour pressure inside the cells of plant samples accelerating cell rupture. During the rupture process, a rapid exudation of the chemical substance within the cells into the surrounding solvents took place (Fig. 6D) (Zhang et al., 2008). During UAE, severe damage on cell walls was observed due to acoustic cavitation (Fig. 6C). The UAE extraction allows the solvent to penetrate cell walls, and the bubbles produced by acoustic cavitation aid in the disruption of the cell wall, which then releases active ingredients (Xia et al., 2011). Treatment of the Myrtle leaves with MAE and UAE likely initiated cell rupture and damage, which

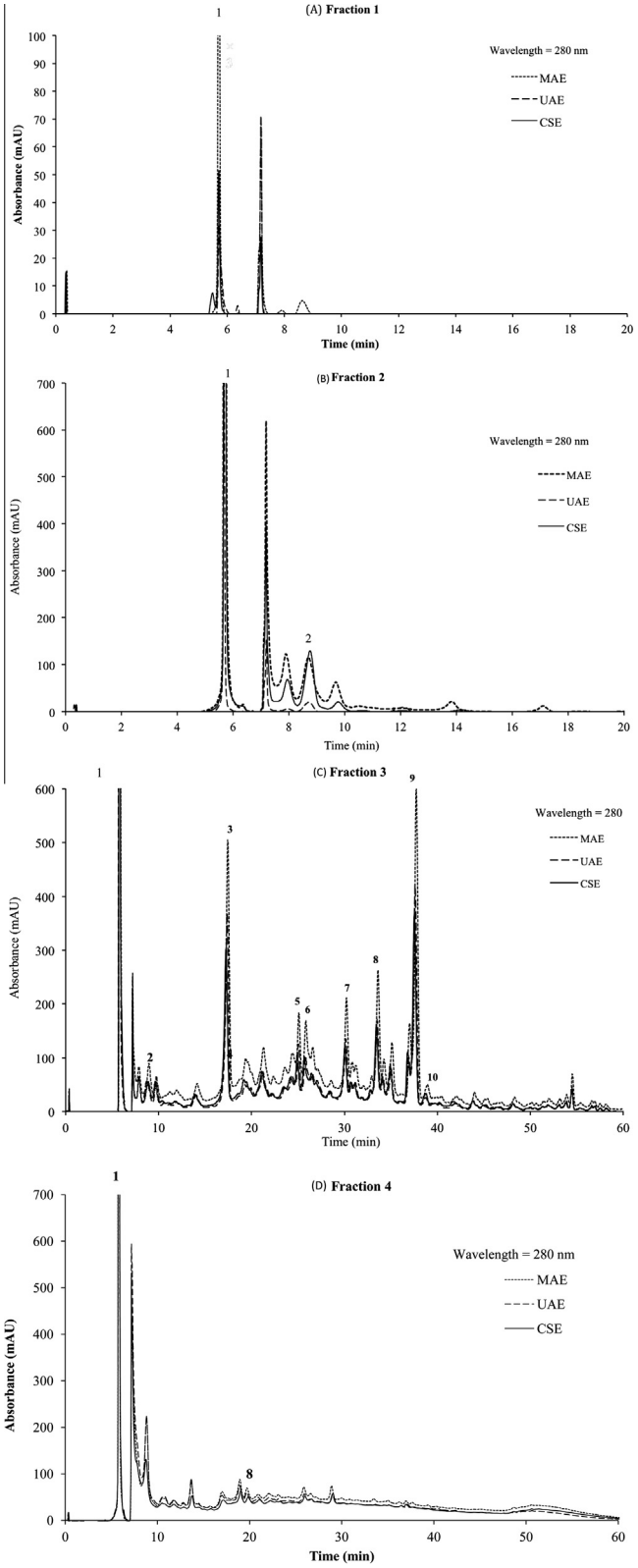


**Fig. 5.** Chromatograms of phenolic acids in different fractions (A: fraction 1; B: fraction 2; C: fraction 3; D: fraction 4) of conventional-solvent extracted (CSE), ultrasound-assisted extracted (UAE), and microwave-assisted extracted (MAE) *M. communis* leaves. Elution times and maximum absorbance of individual phenolic acids were determined using a reverse phase C18 column in HPLC. The mobile phase A was a mixture of 6:94 (v/v) acetic acid in distilled water and mobile phase B consisted of 100% HPLC grade acetonitrile. The solvent gradient in volume ratios was as follows: 0–40 min, 0–25% B; 40–80 min, 25–85% B; 80–90 min, 85–100% B; 90-95 min, 100% B.

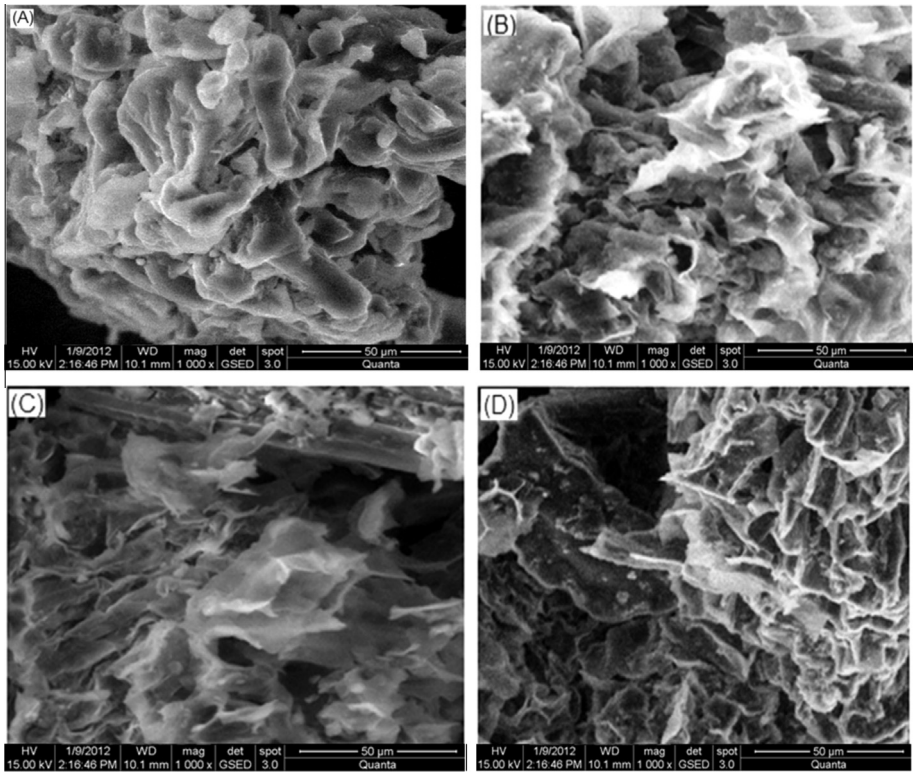


**Fig. 6.** Scanning electron microscopic images of residues in the extraction of untreated *Myrtus communis* leaves (A), conventional-solvent extracted (CSE) leaves (B), ultrasound-assisted extracted (UAE) leaves (C), and microwave-assisted extracted (MAE) leaves (D).

allowed more of the polyphenolic compounds from the powder to be extracted by the solvent. This process is quite different from CSE, which relies on the diffusion of the solvent into the solid matrix and extraction of the components by solubilization (Ballard, Mallikarjunan, Zhou, & O'Keefe, 2010). Therefore, the yield of total phenolics, using CSE, was lower than that observed using MAE and UAE.

## 5. Conclusion

*M. communis* leaves are rich in phytochemicals with high antioxidant activity. However, from the industrial point of view, application of MAE for extraction bioactive compounds from plant material, mathematical models are required to optimise and predict the process in order to replace the conventional extraction methods. Appropriate and optimised processing conditions, such as extraction are required for efficient recovery and cost effectiveness when used on an industrial scale. The present work established an improved and optimised procedure for extracting polyphenols from myrtus leaves using MAE method. It was found that the extraction time of phenolic compounds from *M. communis* leaves extracted using MAE was about 14 and 15 times lower than the UAE and traditional extraction method, respectively. Further studies concerning benefits of polyphenols from the myrtle leaves are required before large scale utilisation is recommended.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.06.066.

## References

Ahmad, J., & Langrish, T. (2012). Optimisation of total phenolic acids extraction from mandarin peels using microwave energy: The importance of the Maillard reaction. *Journal of Food Engineering, 109*(1), 162–174.

Ballard, T. S., Mallikarjunan, P., Zhou, K., & O'Keefe, S. (2010). Microwave-assisted extraction of phenolic antioxidant compounds from peanut skins. *Food Chemistry, 120*(4), 1185–1192.

Barboni, T., Venturini, N., Paolini, J., Desjobert, J., Chiaramonti, N., & Costa, J. (2010). Characterisation of volatiles and polyphenols for quality assessment of alcoholic beverages prepared from Corsican *Myrtus communis* berries. *Food Chemistry, 122*(4), 1304–1312.

Camel, V. (2000). Microwave-assisted solvent extraction of environmental samples. *TrAC, Trends in Analytical Chemistry, 19*(4), 229–248.

Chan, C., Yusoff, R., Ngoh, G., & Kung, F. W. (2011). Microwave-assisted extractions of active ingredients from plants. *Journal of Chromatography A, 1218*(37), 6213–6225.

Chen, W., Wang, W., Zhang, H., & Huang, Q. (2012). Optimization of ultrasonic-assisted extraction of water-soluble polysaccharides from *Boletus edulis* mycelia using response surface methodology. *Carbohydrate Polymers, 87*(1), 614–619.

Dahmoune, F., Boulekbache, L., Moussi, K., Aoun, O., Spigno, G., & Madani, K. (2013). Valorization of Citrus limon residues for the recovery of antioxidants: Evaluation and optimization of microwave and ultrasound application to solvent extraction. *Industrial Crops and Products, 50*, 77–87.

Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., & Mérillon, J. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural and Food Chemistry, 57*(5), 1768–1774.

Gallo, M., Ferracane, R., Graziani, G., Ritieni, A., & Fogliano, V. (2010). Microwave assisted extraction of phenolic compounds from four different spices. *Molecules, 15*(9), 6365–6374.

Hayat, K., Hussain, S., Abbas, S., Farooq, U., Ding, B., Xia, S., et al. (2009). Optimized microwave-assisted extraction of phenolic acids from citrus mandarin peels and evaluation of antioxidant activity *in vitro*. *Separation and Purification Technology, 70*(1), 63–70.

Hossain, M. B., Brunton, N. P., Patras, A., Tiwari, B., O'Donnell, C., Martin-Diana, A. B., et al. (2012). Optimization of ultrasound assisted extraction of antioxidant compounds from marjoram (*Origanum majorana* L.) using response surface methodology. *Ultrasonics Sonochemistry, 19*(3), 582–590.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence

reader in 96-well format. *Journal of Agricultural and Food Chemistry, 50*(16), 4437–4444.
Jaramillo-Flores, M., Gonzalez-Cruz, L., Cornejo-Mazon, M., Dorantes-Alvarez, L., Gutiérrez-López, G., & Hernández-Sánchez, H. (2003). Effect of thermal treatment on the antioxidant activity and content of carotenoids and phenolic compounds of cactus pear cladodes (*Opuntia ficus-indica*). *Food Science and Technology International, 9*(4), 271–278.
Jerman, T., Trebše, P., & Mozetič Vodopivec, B. (2010). Ultrasound-assisted solid liquid extraction (USLE) of olive fruit (*Olea europaea*) phenolic compounds. *Food Chemistry, 123*(1), 175–182.
Jun, X., Deji, S., Ye, L., & Rui, Z. (2011). Comparison of in vitro antioxidant activities and bioactive components of green tea extracts by different extraction methods. *International Journal of Pharmaceutics, 408*(1), 97–101.
Karazhiyan, H., Razavi, S., & Phillips, G. O. (2011). Extraction optimization of a hydrocolloid extract from cress seed (*Lepidium sativum*) using response surface methodology. *Food Hydrocolloids, 25*(5), 915–920.
Li, H., Deng, Z., Wu, T., Liu, R., Loewen, S., & Tsao, R. (2012). Microwave-assisted extraction of phenolics with maximal antioxidant activities in tomatoes. *Food Chemistry, 130*(4), 928–936.
Lou, Z., Wang, H., Zhu, S., Zhang, M., Gao, Y., Ma, C., et al. (2010). Improved extraction and identification by ultra performance liquid chromatography tandem mass spectrometry of phenolic compounds in burdock leaves. *Journal of Chromatography A, 1217*(16), 2441–2446.
Luthria, D. L. (2008). Influence of experimental conditions on the extraction of phenolic compounds from parsley (*Petroselinum crispum*) flakes using a pressurized liquid extractor. *Food Chemistry, 107*(2), 745–752.
Makkar, H., Blümmel, M., & Becker, K. (1995). Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in in vitro techniques. *British Journal of Nutrition, 73*(06), 897–913.
Messaoud, C., Laabidi, A., & Boussaid, M. (2012). *Myrtus communis* L. infusions: The effect of infusion time on phytochemical composition, antioxidant, and antimicrobial activities. *Journal of Food Science, 77*(9), C941–C947.
Pan, Y., He, C., Wang, H., Ji, X., Wang, K., & Liu, P. (2010). Antioxidant activity of microwave-assisted extract of *Buddleia officinalis* and its major active component. *Food Chemistry, 121*(2), 497–502.
Pérez-Serradilla, J., & Luque de Castro, M. (2011). Microwave-assisted extraction of phenolic compounds from wine lees and spray-drying of the extract. *Food Chemistry, 124*(4), 1652–1659.
Quettier-Deleu, C., Gressier, B., Vasseur, J., Dine, T., Brunet, C., Luyckx, M., et al. (2000). Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *Journal of Ethnopharmacology, 72*(1–2), 35–42.
Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 26*(9), 1231–1237.
Romani, A., Campo, M., & Pinelli, P. (2012). HPLC/DAD/ESI-MS analyses and anti-radical activity of hydrolyzable tannins from different vegetal species. *Food Chemistry, 130*(1), 214–221.
Song, J., Li, D., Liu, C., & Zhang, Y. (2011). Optimized microwave-assisted extraction of total phenolics (TP) from *Ipomoea batatas* leaves and its antioxidant activity. *Innovative Food Science & Emerging Technologies, 12*(3), 282–287.
Spigno, G., & De Faveri, D. (2009). Microwave-assisted extraction of tea phenols: A phenomenological study. *Journal of Food Engineering, 93*(2), 210–217.
Spigno, G., Tramelli, L., & De Faveri, D. M. (2007). Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *Journal of Food Engineering, 81*(1), 200–208.
Tattini, M., Remorini, D., Pinelli, P., Agati, G., Saracini, E., Traversi, M. L., et al. (2006). Morpho-anatomical, physiological and biochemical adjustments in response to root zone salinity stress and high solar radiation in two Mediterranean evergreen shrubs, *Myrtus communis* and *Pistacia lentiscus*. *New Phytologist, 170*(4), 779–794.
Wannes, W. A., Mhamdi, B., Sriti, J., & Marzouk, B. (2010). Glycerolipid and fatty acid distribution in pericarp, seed and whole fruit oils of *Myrtus communis* var. *italica*. *Industrial Crops and Products, 31*(1), 77–83.
Xia, E., Ai, X., Zang, S., Guan, T., Xu, X., & Li, H. (2011). Ultrasound-assisted extraction of phillyrin from *Forsythia suspensa*. *Ultrasonics Sonochemistry, 18*(2), 549–552.
Yang, Y., Li, J., Zu, Y., Fu, Y., Luo, M., Wu, N., et al. (2010). Optimisation of microwave-assisted enzymatic extraction of corilagin and geraniin from *Geranium sibiricum* Linne and evaluation of antioxidant activity. *Food Chemistry, 122*(1), 373–380.
Zhang, G., Hu, M., He, L., Fu, P., Wang, L., & Zhou, J. (2013). Optimization of microwave-assisted enzymatic extraction of polyphenols from waste peanut shells and evaluation of its antioxidant and antibacterial activities in vitro. *Food and Bioproducts Processing, 91*(2), 158–168.
Zhang, Z., Li, D., Wang, L., Ozkan, N., Chen, X. D., Mao, Z., et al. (2007). Optimization of ethanol–water extraction of lignans from flaxseed. *Separation and Purification Technology, 57*(1), 17–24.
Zhang, B., Yang, R., & Liu, C. (2008). Microwave-assisted extraction of chlorogenic acid from flower buds of *Lonicera japonica* Thunb. *Separation and Purification Technology, 62*(2), 480–483.