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Ultrasound extraction optimization for bioactive molecules from *Eucalyptus globulus* leaves through antioxidant activity

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ABSTRACT

Antioxidant products present a very high added value and are demanded in the market. The optimization of their extraction is a high-stakes matter for both economic and environmental points of view. Ultrasound extraction has been considered one of the most promising methods, so the relative importance of key parameters may have decisive economic significance. For this reason, different parameters that have influence on the extraction capacity such as ultrasound power, time, temperature, pH and % ethanol in water have been studied to know the relationships between the independent parameters and their influence on the extraction from *Eucalyptus globulus* leaves. An experimental Box-Behnken factorial design and subsequent analysis by neural networks have been used. The relative influence of each parameter varies according to the nature of the extracted compound. In this regard, the higher capacity of extraction of the selected antioxidant compounds by means of the variation of the operation conditions can be facilitated. For all the studied compounds, temperature has been the most important parameter for their extraction. The relative content (%) of bioactive compounds (terpenes) in the optimized *Eucalyptus globulus* extract has been performed by GC–MS analysis.

1. Introduction

Consumers are increasingly looking for products that offer health benefits. To meet these needs, industry must find ways to make products that adequately meet these expectations for both health and the environment point of view. In this sense, antioxidant substances are one of the most demanded products by consumers [1].

In order to increase the lifetime of packaged foods and prevent product degradation, the industry uses predominantly synthetic antioxidants. They offer an adequate response in people, but their use in food implies, in many occasions, the loss of the organoleptic quality and nutritional value of the food. Moreover, these synthetic antioxidants have been questioned because of their possible toxicity and collateral effects [2]. In this sense, natural antioxidants are the best alternative.

Diverse plant species, such as eucalyptus, cashew, camphor tree, poplar, drumstick tree, olive have been evaluated in relation to their capacity and antioxidant components [3–6]. Eucalyptus are recognized as one of the fastest growing trees. Therefore, this genus is widespread in the world, occupying>20 million hectares [7]. Biomass percentages (w/w) for Eucalyptus stands among 78–82% for wood and 11–20% for leaves and thin branches have been obtained [8,9].

The interest in antioxidants, mainly phenolic compounds, present in plants has increased significantly in the last decade. The leaves and fine branches of eucalyptus have a large number of phenolic compounds that can be valorized by different industrial sectors, among which the pharmaceutical, sanitary, agricultural and food industries could be highlighted [3–10]. Recently, several studies have been conducted using *Eucalyptus globulus* leaves to study the potential capacity of these plants as antioxidants [3,11–13].

Various chromatographic techniques such as high-performance liquid chromatography (HPLC) [3,11,14,15] and gas chromatography (GC) coupled to mass spectrometry (MS) [11,12,14,16–22] to determine the composition of compounds extracted from biomass have been used. In this regard, the presence of terpenes [11,12,14,17–21] and other bioactive compounds with antioxidant properties in the leaves and wood of different eucalyptus species have been determined. Nevertheless, depending on the selected eucalyptus species and the extraction method used, different extracted quantities have been found. In *Eucalyptus globulus* leaves, 1, 8-cineole (Eucalyptol) were the main terpenes in methanol extracts and essential oil [11,12,21].

The great increase of applications found for the products obtained from eucalyptus has achieved the development of different efficient

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Table 1
Experimental Box-Behnken factorial design used and values of total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity and ABTS radical scavenging activity found under the selected experimental design.

Run	Independent Variables					Total Phenolic and Flavonoid Contents and Antioxidant Activities[e]			
	pH	T[a] (°C)	EW[b] (% v/v)	P[c] (W)	t[d] (min)	TPC[f]	TFC[g]	DPPH[h]	ABTS[i]
1	−1(3)	−1 (30)	−1(15)	−1(40)	1(15)	52 ± 2	25 ± 1	71 ± 4	70 ± 3
2	−1(3)	−1(30)	−1(15)	1(120)	−1(5)	66 ± 3	8.2 ± 0.4	68 ± 4	77 ± 3
3	−1(3)	−1(30)	1(25)	−1(40)	−1(5)	49 ± 2	6.2 ± 0.3	53 ± 3	57 ± 3
4	−1(3)	−1(30)	1(25)	1(120)	1(15)	86 ± 3	11 ± 1	91 ± 4	98 ± 4
5	−1(3)	1(50)	−1(15)	−1(40)	1(15)	22 ± 1	2.7 ± 0.1	14 ± 1	12 ± 1
6	−1(3)	1(50)	−1(15)	1(120)	1(15)	67 ± 3	8.4 ± 0.3	79 ± 4	84 ± 4
7	−1(3)	1(50)	1(25)	−1(40)	1(15)	89 ± 3	11 ± 1	90 ± 3	94 ± 4
8	−1(3)	1(50)	1(25)	1(120)	−1(5)	37 ± 2	6.4 ± 0.3	42 ± 2	39 ± 2
9	1(5)	−1(30)	−1(15)	−1(40)	1(15)	24 ± 1	3.0 ± 0.1	21 ± 1	28 ± 1
10	1(5)	−1(30)	−1(15)	1(120)	1(15)	56 ± 2	7.0 ± 0.3	54 ± 3	72 ± 4
11	1(5)	−1(30)	1(25)	−1(40)	1(15)	60 ± 3	7.5 ± 0.4	84 ± 4	83 ± 4
12	1(5)	−1(30)	1(25)	1(120)[a]	1(15)	90 ± 4	12 ± 0.5	104 ± 4	107 ± 4
13	1(5)	1(50)	−1(15)	−1(40)	1(15)	115 ± 4	14 ± 0.7	65 ± 3	66 ± 3
14	1(5)	1(50)	−1(15)	1(120)	−1(5)	36 ± 2	4.6 ± 0.2	25 ± 1	25 ± 1
15	1(5)	1(50)	1(25)	−1(40)	−1(5)	47 ± 2	5.8 ± 0.3	41 ± 2	43 ± 2
16	1(5)	1(50)	1(25)	1(120)	1(15)	60 ± 3	7.5 ± 0.4	59 ± 2	47 ± 2
17	0(4)	0(40)	0(30)	0(80)	0(10)	46 ± 2	5.7 ± 0.3	32 ± 1	28 ± 1
18	0(4)	0(40)	0(30)	0(80)	0(10)	40 ± 2	5.1 ± 0.3	36 ± 2	44 ± 2
19	0(4)	0(40)	0(30)	0(80)	0(10)	36 ± 2	4.5 ± 0.2	24 ± 1	22 ± 2
20	−2(2)	0(40)	0(30)	0(80)	0(10)	48 ± 2	8.2 ± 0.3	45 ± 2	52 ± 2
21	2(6)	0(40)	0(30)	0(80)	0(10)	40 ± 2	6.2 ± 0.3	37 ± 2	44 ± 2
22	0(4)	−2(20)	0(30)	0(80)	0(10)	44 ± 2	5.4 ± 0.3	43 ± 2	62 ± 3
23	0(4)	2(60)	0(0)	0(80)	0(10)	104 ± 5	13 ± 0.6	107 ± 5	93 ± 5
24	0(4)	0(40)	−2(30)	0(80)	0(10)	43 ± 2	5.4 ± 0.3	43 ± 2	58 ± 3
25	0(4)	0(40)	2(60)	0(80)	0(10)	76 ± 4	9.7 ± 0.4	83 ± 4	71 ± 3
26	0(4)	0(40)	0(30)	−2(0)	0(10)	32 ± 1	3.9 ± 0.2	39 ± 2	42 ± 3
27	0(4)	0(40)	0(30)	2(160)	0(10)	49 ± 2	6.1 ± 0.3	44 ± 2	49 ± 2
28	0(4)	0(40)	0(30)	0(80)	−2(1)	36 ± 2	4.5 ± 0.2	12 ± 1	16 ± 1
29	0(4)	0(40)	0(30)	0(80)	2(20)	53 ± 2	6.6 ± 0.3	43 ± 2	54 ± 3

[a] Temperature (T); [b]% ethanol in water (EW); [c]Ultrasound power (P); [d]Ultrasoundtime (t); [e]Each value is the average of three samples (standard deviation < 5%);[f]TPC: mg gallic acid g^{-1} of dry leaves (dw.); [g]TFC: mg epicatechin g^{-1} of dry leaves(d.w.); [h]DPPH scavenging activity: mg ascorbic acid g^{-1} of dry leaves (d.w.);[i]ABTS scavenging activity: mg ascorbic acid g^{-1} of dry leaves (d.w.).

extraction systems, and we can highlight conventional extraction by reflux with different solvents, microwave assisted extraction and ultrasonic assisted extraction, as well as several associations among different techniques [23,24]. However, there is an increasing environmental interest to reduce the use of organic solvents using green technologies. They allow the development of efficient systems, but preventing negative influences on the environment. In this sense, ultrasound extraction is a promising technique that has gained more and more acceptance [25]. This technology has a variety of economic and environmental advantages since it minimizes the use of solvents and chemical additives, reduces time consumption, facilitating the separation and reusing of the components.

Several authors have demonstrated the effectiveness of ultrasound in the extraction of antioxidants under different operating parameters [25,26]. This technology also allows to increase the yield, decrease the time and increase the extraction of antioxidant and antimicrobial compounds [27]. Although this technique may decrease the stability of the extracted components, there are studies focused on the solution of these problems. Few of them have showed the relative importance of all the variables (individually or in combination) involved in the extraction process and their influence on the antioxidant capacity of the extracted components. Major technological efforts for their large-scale industrial application are being successfully pursued [28].

The relative influence of the main variables in ultrasound extraction process [ultrasound power (0–160 W) and time (1–20 min)] and other involved operation variables [pH (2–6), temperature (20–60 °C) and % ethanol in water (0–60% v/v)] have been examined and optimized in this work by using an experimental Box-Behnken factorial design. To analyze, model and determine the influence of the studied parameters that maximize both the extraction of bioactive compounds and antioxidant activity in *Eucalyptus globulus* leaves, an adaptive network-based fuzzy inference system (ANFIS) has been used.

2. Materials and methods

2.1. Raw material

Fresh leaves of *Eucalyptus globulus* from La Rábida Campus (University of Huelva) were collected. The leaves were taken to the laboratory, then washed and cut to 5 cm fractions by hand. An average moisture of 75% (±4%) has been found. 100 g of fresh and clean *Eucalyptus globulus* leaves were used for each test.

2.2. Ultrasound extraction

The extraction was carried out using an ultrasonic homogenizer (Cobos, Ultrasonic Baths Power Sonic 510, made by Hwahin Technology, Seoul, Korea) with a maximum frequency of 40 kHz. The power (0–160 W) was controlled, depending on the experimental design, to establish the output power of the ultrasounds. To maintain the selected initial temperature, a Julabo Corio C Heating Immersion Circulators has been used. Ethanol, water and various ratio of their mixture have been used as extraction solvents. The pH (initial pH 5.5) was fixed by means of hydrochloric acid Fresh *Eucalyptus globulus* leaves were added at a fixed ratio of 1:10 (w/v, dry weight) [29]. 10 g of fresh *Eucalyptus globulus* leaves and 100 mL of solvent were used for the extraction optimization. After to extraction, the extract was filtered through a 0.2 mm filter and stored at −20 °C until analysis.

On the other hand, as a method of control of the efficiency of the ultrasound extraction, separate experiences, different from the selected experimental design, for each of the selected compounds have been carried out (duplicate). In these control experiments, the same independent variables (pH, temperature, % ethanol in water and ultrasound time) and the same values, as those found for the maximum extraction of each of the compounds studied, although without ultrasound, have been

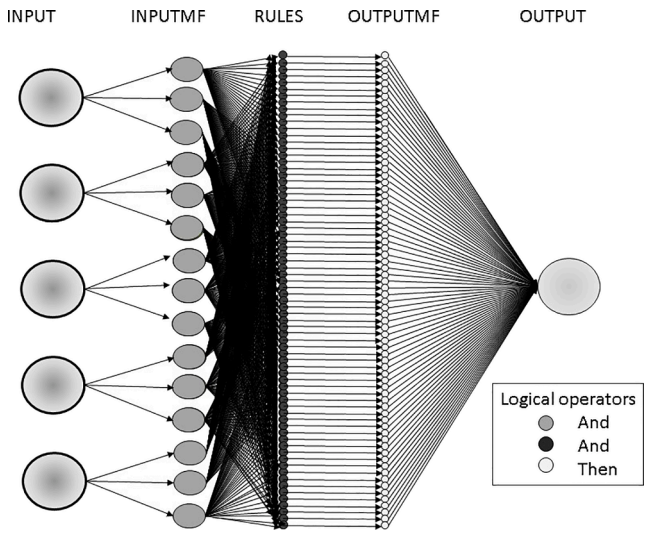

Fig. 1. ANFIS model structure used in this study. Where: INPUT: Raw independent variables. INPUTMF: Data in the Membership Function. OUTPUTMF: Data obtained for each Membership Function. OUTPUT: Dependent variable data.

applied. This difference, in percentage, for the maximum extraction with and without ultrasound will provide us with a measure of the efficiency of this process for each compound studied.

2.3. Experimental design

The effect of ultrasound extractions depended strongly on the operating conditions [30]. Several authors have studied various ultrasound power and levels of the extraction parameters, such as, extraction time, temperature, liquid–solid ratio and % ethanol in water [25,26,31].

A Box-Behnken factorial design (Table 1) has been used to examine the influence of the independent operational variables [pH, temperature (T), % ethanol in water (EW), ultrasound power (P) and ultrasound time (t)] on the characteristic of liquors extracted: total phenolic content (calculated in terms of gallic acid units, TPC), total flavonoid content (calculate in terms of epicatechin units, TFC) and the antioxidant activity (using both the free radical 2,2-diphenyl-1-picrylhydrazyl, DPPH and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid), ABTS methods).

The total number of experiments required for the selected independent variables at three levels was 29. In order to facilitate a direct comparison of the calculated coefficients (related to the effects of the individual independent variables on the response variable), the independent variables values were normalized from −1 to + 1 by using Eq. (1).

$$X_n = \frac{X - \overline{X}}{(X_{max} - X_{min})/2} \quad (1)$$

where X_n is the normalized value of independent variables; X is the absolute experimental value of the variable concerned; $\overline{X}$ is the mean of all the experimental values for the independent variable and X_{max} and X_{min} are the maximun and minimun value of the independent variable, respectively. In our case, they were +1 when pH, temperature, % ethanol in water, ultrasound power and ultrasound time were 6, 60C, 60% v/v, 120 W and 15 min, respectively.

2.4. Statistical analysis by adaptative neural fuzzy inference system (ANFIS)

Diffuse modelling [32] is a widely used tool to describe the behavior of complex non-linear systems. This type of modelling has been previously applied to the simulation and control of many chemical and biological processes. In addition, neural networks (NN) are another tool used for the modelling of complex systems. Based on the first-order Suggeno-diffuse model, Jang [33] combined the advantages of both systems offering a new tool, the adaptive network-based fuzzy inference system (ANFIS). The NN paradigm is based on the use of a multi-layered retropropagation network. The detailed architecture of the ANFIS and its learning procedure can be found in the literature [33].

The mathematical equation which responds to different rules is:

$$y_e = \frac{\sum y_l \ R_l}{\sum R_l} \quad (2)$$

y_e = output variable estimate value
m = number of rules
y_l = defuzzifier.
R_l = product of the selected membership functions.

Eq. (3) is the result of simplifying Eq. (2) for the variables studied (29 fuzzy rules, three levels for each independent variable):

Table 2
Calculated plot of each dependent variable versus each independent one constructed by changing all the independent variables between the normalized values from –1 to +1 in obtained ANFIS equations and the influence in % of each independent variables on the dependent variables.

	T	pH	EW	P	t
TPC	18.08%	17.56%	18.79%	17.49%	21.19%
TFC	18.29%	16.84%	17.90%	16.71%	18.26%
DPPH	17.84%	17.07%	16.95%	17.15%	16.99%
ABTS	20.17%	18.55%	18.60%	18.70%	18.48%

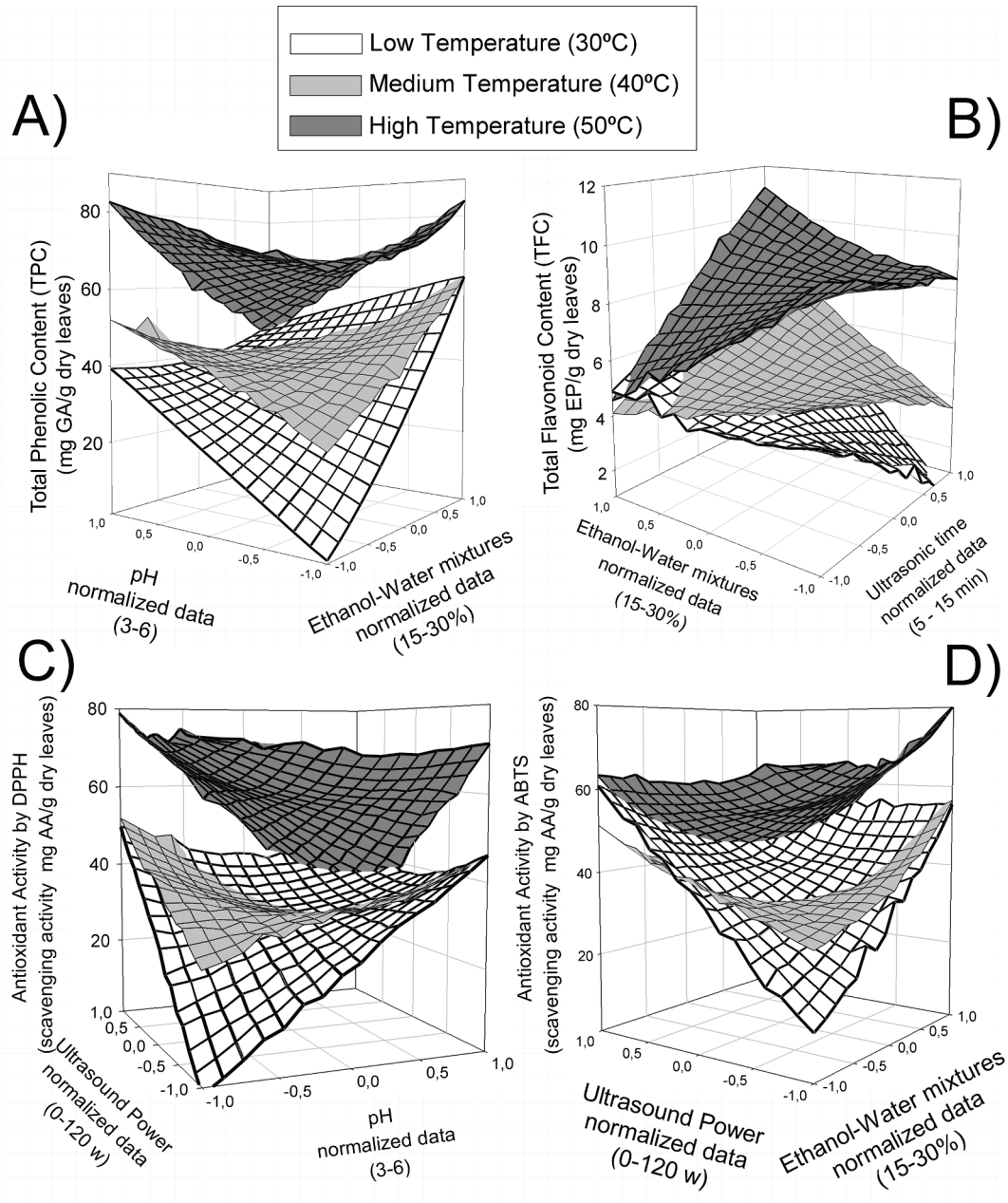

Fig. 2. Total antioxidant compounds as function of main extracted parameters.

$$y_e = \frac{\sum_{l=1}^{29} c_l \left[\prod_{i=1}^{3} x_i\right]}{\sum_{l=1}^{29} \left[\prod_{i=1}^{3} x_i\right]} \quad (3)$$

For this study, where "c_1", is a constant (defuzzifier). In that form, y_l represents the linear behavior of the system in the conditions of independent variables defined by R_l.

The ANFIS model structure used for the independent variables is shown in Fig. 1. Moreover, the membership functions, constants and parameters (supplementary material) has been calculated by using the ANFIS tool in the Matlab (Fuzzy Logic Toolbox Version 2.2.2, Neural Network Toolbox Version 4.0.6) software suite.

2.5. Standards and chemicals reagents

Gallic acid (GA), ascorbic acid (AA) and epicatechin (EP) of the highest purity (>98%), were supplied from Sigma-Aldrich (Sigma-Aldrich, Gillingham, U.K.). Folin-Ciocalteu reagent, DPPH, ABTS, aluminum chloride and absolute ethanol analytical-reagent grade were obtained from Sigma-Aldrich. Sodium carbonate, sodium nitrite, sodium hydroxide, potassium persulfate and methanol were provided by Merck (Darmstadt, Germany). Ultra-pure Milli-Q water (18 MΩ cm) (Millipore, Bedford, MA, USA) was used.

2.6. Determination of total phenolic (TPC)

The TPC measure sample reducing capacity [34] was determined spectrophotometrically according to the Folin-Ciocalteu reactive [35]. TPC measure sample 1 mL of extract was mixed with 2.5 mL of the Folin-Ciocalteu reagent (1:10) and 2 mL of 7.5% w/v Na_2CO_3 solution. The mixture was kept at 45 °C for 15 min and, subsequently, for 30 min at room temperature. Absorbance was measured at 765 nm (Genesys 10 UV spectrophotometer from Thermo Electron Corp.) with quartz

Table 3
Chemical composition and relative content (%) of optimized *Eucalyptus globulus* extract determined by GC/MS.

Peak	Compound name [a]	Chemical formula	Molecular Weight	Retention time (min)	relative content (%)
1	Eucalyptol	$C_{10}H_{18}O$	154.13	14.129	67.29
2	Camphenol	$C_{10}H_{16}O$	152.12	16.513	0.32
3	Pinocarveol	$C_{10}H_{16}O$	152.12	16.842	11.11
4	Pinocarvone	$C_{10}H_{14}O$	150.10	17.374	11.33
5	*trans*-p-mentha-1(7) 0.8-dien-2-ol	$C_{10}H_{16}O$	152.12	17.897	0.91
6	*cis*-p-mentha-1(7) 0.8-dien-2-ol	$C_{10}H_{16}O$	152.12	18.743	0.70
7	*exo*-2-Hydroxycineole	$C_{10}H_{18}O_2$	170.13	20.860	1.15
8	α-Terpineol acetate	$C_{12}H_{20}O_2$	154.13	20.987	1.39
9	Alloaromadendrene	$C_{15}H_{24}$	204.19	22.738	0.07
10	Epiglobulol	$C_{15}H_{26}0$	222.20	24.685	0.51
11	Viridiflorol	$C_{15}H_{26}O$	222.20	25.077	5.22

[a] Compounds listed in order of elution.

cuvettes of 1.0 cm path length. Calibration curves for gallic acid standards were obtained in the ranges of 0–40 mg L^{-1} ($r > 0.9986$), respectively. TPC concentration was expressed as milligrams of gallic acid per gram of dry leaves.

2.7. Determination of flavonoid content (TFC)

TFC was determined using a colorimetric test based on the formation of an aluminum-flavonoid complex [36]. 1 mL of extract was added to 300 μL of a 5% $NaNO_2$ solution. These mixtures were stored at room temperature for 5 min, and then 300 μL of 10% $AlCl_3$ solution was added. The samples were again stored at room temperature for 6 min. Then, 2 mL of NaOH 1 M and 2.4 mL of ultrapure water were added. The absorbance at 510 nm of the pink solutions was measured. Epicatechin was used as the standard compound. The calibration curve was recorded in the range of 0–80 mg L^{-1} ($r > 0.9999$) and the results for TFC were expressed as milligrams of epicatechin per gram of dry leaves.

2.8. Determination of the antioxidant activity

Antioxidant activity of *Eucalyptus globulus* leaves samples was measured using two assays of radical scavenging activity: the 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

For the measurement of antioxidant capacity using ABTS as oxidant, a solution was prepared by mixing an ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate [37]. This mixture was allowed to stand for 12 h at room temperature in the dark until reaching a stable oxidative state. This solution was subsequently diluted in water in 1:40 ratio (0.096 mg mL^{-1}). Standard solutions of ascorbic acid in the concentration range of 1–12 mg L^{-1} were prepared. 1 mL of the standard solutions were mixed with 1 mL of the ABTS 0.096 mg mL^{-1} solution. The mixtures were kept 10 min in the dark at room temperature, and the absorbance was recorded at 734 nm. Samples were diluted 1:200 with water. 1 mL of this solution was mixed with 1 mL of the ABTS 0.096 mg mL^{-1}, and proceeded in the same way as the standards. The antioxidant capacity was expressed as milligrams of ascorbic acid per gram of dry leaves.

The method Brand-Williams et al. [38] was also used. It was based on the measurement of the capacity of the samples and standards to scavenge the DPPH radical. DPPH (2.11 mg mL^{-1}) was dissolved in 100% methanol. Standard solutions of ascorbic acid (in the range of 0–12 mg L^{-1}) were prepared. 1 mL of the standards were mixed with 1 mL of the DPPH methanolic solution to stablished the percentage of diminution of absorbance of radical DPPH for each standard concentration. The mixtures were kept 30 min at room temperature, and the absorbance was measured at 515 nm. Samples were diluted 1:200 with water. 1 mL of the solution was mixed with 1 mL of the DPPH 2.32 mg mL^{-1}. The antioxidant capacity was expressed as milligrams of ascorbic acid per gram of dry leaves.

2.9. Gas chromatography analysis (GC–MS)

A 200 μL of extract E. globulus leaves was placed in a 40 mL glass vial containing 10 mL of water. Then, a stir bar (20 mm length × 0.5 mm film thickness) coated with 47 μL of PDMS from Gerstel (Mulheim an der Ruhr, Germany) was placed in the solution and stirring at 900 rpm for 60 min at room temperature using Gerstel magnetic stirrer. Next, stir bar were removed from vial, rinsed with Milli-Q water, dried with a paper tissue and introduction in glass tube of the thermal desorption system unit (TD-20, Shimadzu, Japan) coupled to the GC–MS/MS (GCMSQP6030 Ultra, Shimadzu, Japan). Bioactive compounds were desorbed at 280 °C for 15 min, the derived analytes being preconcentrated on the cold trap at −20 °C and then thermally desorbed at 300 °C for 8 min. The analyses were carried out using a HP-5 MS column (60 m, 0.25 mm I.D. 0.25 μm film thickness, J&W Scientific, Agilent Technologies, USA). The column was kept at 60 °C for 7 min, ramped at 8 °C min^{-1} to 280 °C, held for 6 min. Helium at constant flow rate of 1.3 mL min^{-1} was used as the carrier gas. The temperatures of the transfer line and ion source were maintained at 280 and 230 °C, respectively. Terpenes compounds were identified by comparison of the mass spectra with those of the database of NIST11 library.

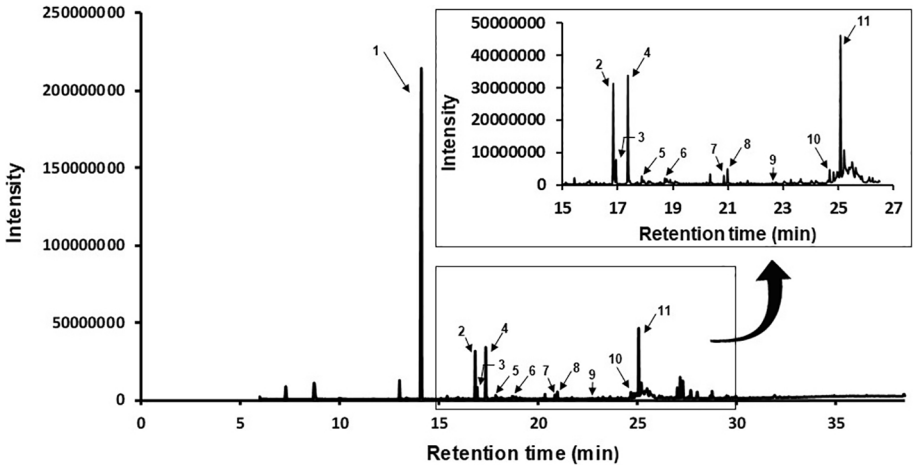

Fig. 3. GC/MS chromatogram of the optimized *Eucalyptus globulus* extract.

3. Results and discussion

3.1. ANFIS model and influence of independent variables.

A total of 29 experiments considering pH, temperature (T), % ethanol in water (EW), ultrasound power (P) and ultrasound time (t) were carried out. The normalized values of independent variables and properties of the extracts obtained after ultrasound treatments are shown in Table 1.

Moreover, $R_i = \pi x_i$, by the product of five membership functions (5 independent variables) is defined. In that form, the combinations of membership functions are:

$R_1 = pH_{-1}\ T_{-1}\ EW_{-1}\ UP_{-1}\ t_{+1}$	(Eq. 4)	$R_2 = pH_{-1}\ T_{-1}\ EW_{+1}\ UP_{-1}\ t_{-1}$	(Eq. 5)
$R_3 = pH_{-1}\ T_{+1}\ EW_{-1}\ UP_{-1}\ t_{-1}$	(Eq. 6)	$R_4 = pH_{-1}\ T_{-1}\ EW_{+1}\ UP_{+1}\ t_{+1}$	(Eq. 7)
$R_5 = pH_{-1}\ T_{+1}\ EW_{-1}\ UP_{-1}\ t_{-1}$	(Eq. 8)	$R_6 = pH_{-1}\ T_{+1}\ EW_{-1}\ UP_{-1}\ t_{+1}$	(Eq. 9)
$R_7 = pH_{-1}\ T_{+1}\ EW_{+1}\ UP_{-1}\ t_{+1}$	(Eq. 10)	$R_8 = pH_{-1}\ T_{+1}\ EW_{+1}\ UP_{+1}\ t_{-1}$	(Eq. 11)
$R_9 = pH_{+1}\ T_{-1}\ EW_{-1}\ UP_{-1}\ t_{-1}$	(Eq. 12)	$R_{10} = pH_{+1}\ T_{-1}\ EW_{-1}\ UP_{+1}\ t_{+1}$	(Eq. 13)
$R_{11} = pH_{+1}\ T_{-1}\ EW_{+1}\ UP_{-1}\ t_{+1}$	(Eq. 14)	$R_{12} = pH_{+1}\ T_{-1}\ EW_{+1}\ UP_{+1}\ t_{-1}$	(Eq. 15)
$R_{13} = pH_{+1}\ T_{+1}\ EW_{-1}\ UP_{-1}\ t_{+1}$	(Eq. 16)	$R_{14} = pH_{+1}\ T_{+1}\ EW_{-1}\ UP_{+1}\ t_{-1}$	(Eq. 17)
$R_{15} = pH_{+1}\ T_{+1}\ EW_{+1}\ UP_{-1}\ t_{-1}$	(Eq. 18)	$R_{16} = pH_{+1}\ T_{+1}\ EW_{+1}\ UP_{+1}\ t_{+1}$	(Eq. 19)
$R_{17} = pH_0\ T_0\ EW_0\ UP_0\ t_0$	(Eq. 20)	$R_{18} = pH_0\ T_0\ EW_0\ UP_0\ t_0$	(Eq. 21)
$R_{19} = pH_0\ T_0\ EW_0\ UP_0\ t_0$	(Eq. 22)	$R_{20} = pH_{-2}\ T_0\ EW_0\ UP_0\ t_0$	(Eq. 23)
$R_{21} = pH_{+2}\ T_0\ EW_0\ UP_0\ t_0$	(Eq. 24)	$R_{22} = pH_0\ T_{-2}\ EW_0\ UP_0\ t_0$	(Eq. 25)
$R_{23} = pH_0\ T_{+2}\ EW_0\ UP_0\ t_0$	(Eq. 26)	$R_{24} = pH_0\ T_0\ EW_{-2}\ UP_0\ t_0$	(Eq. 27)
$R_{25} = pH_0\ T_0\ EW_{+2}\ UP_0\ t_0$	(Eq. 28)	$R_{26} = pH_0\ T_0\ EW_0\ UP_{-2}\ t_0$	(Eq. 29)
$R_{27} = pH_0\ T_0\ EW_0\ UP_{+2}\ t_0$	(Eq. 30)	$R_{28} = pH_0\ T_0\ EW_0\ UP_0\ t_{-2}$	(Eq. 31)
$R_{29} = pH_0\ T_0\ EW_0\ UP_0\ t_{+2}$	(Eq. 32)		

According to ANFIS analysis, the experimental conditions (independent variables) for each dependent variable measured into the fuzzy models, yielded the parameters, constants and membership functions and the mathematical models are obtained (supplementary material). The optimum number of rules has been selected as 29 in this study. The developed ANFIS model produced a low prediction error with correlation coefficient (R) value of 0.98.

Table 2 shows several plots of each dependent variable against each independent one constructed by changing all the independent variables between the normalized values from –1 to +1. At a given value of an independent variable, the magnitude of the difference between the maximum and minimum values of the dependent variable is related to the influence of the independent variables other than that plotted on the variation of the dependent variable concerned. Thus, if the independent variable plotted had no effect, then the difference between the maximum and minimum values of the dependent variable in question would coincide with the height of the rectangle having the range of values of the independent variable plotted, $[(X_{ni})_{max} - (X_{ni})_{min}]$, and the maximum possible difference between the maximum and minimum values of the dependent variable considered, $\{Z(X_{ni})_{max}]_{max} - Z[(X_{ni})_{min}]_{min}\}$, as its bases.

Because the influence of the other variables on the dependent variable considered can vary with each value of the independent variable plotted, the average change in the dependent variable will be given by Eq. (33).

$$\frac{\int_{(X_{ni})_{min}}^{(X_{ni})_{max}} [Z(X_{ni})_{max} - Z(X_{ni})_{min}]\,dX_{ni}}{[(X_{ni})_{max} - (X_{ni})_{min}]} \tag{33}$$

The change in the dependent variable with that in the independent variable plotted can be express as the difference between $\{Z(X_{ni})_{max}]_{max} - Z[(X_{ni})_{min}]_{min}\}$ and the previous expression:

$$DZ_i = \left\{ [Z(X_{ni})_{max}]_{max} - [Z(X_{ni})_{min}]_{min} \right\} - \frac{\int_{(X_{ni})_{min}}^{(X_{ni})_{max}} [Z(X_{ni})_{max} - Z(X_{ni})_{min}]\,dX_{ni}}{[(X_{ni})_{max} - (X_{ni})_{min}]} \tag{34}$$

Table 2 shows the DZi values. These values allow one to weight the relative influences, as percentages, of each independent variable on the variation of each dependent variable. Consequently, the relative influence among the independent variables can be calculated (Table 2). Temperature mainly influenced at high values. The opposite was found for the % ethanol in water and ultrasound power. The pH had its strongest influence at the extreme values (3 and 6). Finally, sonication time had a limited influence.

The TFC of the extracts mainly depended on the temperature, followed by time. DPPH and ABTS were influenced by the temperature followed by ultrasound power. However, the TPC was influenced by time and % ethanol in water. The reasons for the differences in the extraction values could be (i) different extraction kinetics of these components, (ii) presence of some substances in the liquid that could have a synergistic effect for the extraction and (iii) selective degradation of some components under certain chemical conditions [39].

The positive effect of temperature on the extraction yield has been demonstrated (Table 2), although it also depended on the other process parameters used. With increasing extraction time and ultrasound power, a higher extraction capacity was demonstrated for all components. Possibly, this can be explained by the fact that mass transfer is a time-dependent process. On the other hand, at low pH values, the degradation of the extracted components increased with increasing extraction time. Therefore, the extraction time should not exceed 10 min. Although longer ultrasound time will provide a better contact of the solvent with the biomass, it also will cause an excessive degradation, resulting in reduced extraction yield.

3.2. Response surfaces for dependent variables

Different response surfaces have been designed to facilitate the display of the optimum values of the extraction conditions (Fig. 2).

High temperatures were suitable to obtain higher TPC values (Fig. 2-A). Bhuyan et al. [40] found that the temperature, followed by time and power, played an important influence on the extraction efficiency of TPC. High temperatures favored the extraction efficiency of bioactive compounds because of the increase of acoustic cavitation, surface contact area and the decrease of the solvent viscosity and density [41]. All these factors increased the rupture of the cell walls in the plant, releasing several bioactive compounds [42,43]. In addition, the increase of extraction temperature reduced the extraction time.

There was an interdependence between % ethanol in water and pH: for high pH values, phenol content was better extracted with low % ethanol in water. The opposite was observed for low pH values. At high values of pH, —OH groups of TPC can be dissociated, its polarity is increased, and are more soluble in water [44]. Some authors [41,44] suggested that the use of a binary solvent system increased the extraction of bioactive compounds in plants. TPC and TFC are highly soluble in hydroalcoholic solutions, whereas that compounds with antioxidant capacity are more hydrophilic. Several authors [41,44] obtained a higher TPC extraction efficiency when using 50% of EtOH.

Fig. 2-B showed a direct dependence of the TFC extraction on % ethanol in water at low temperatures, but the opposite was found at high temperatures. Temperatures above 70 °C may cause the degradation of

thermolabile compounds such as some flavonoids [41–43]. Moreover, sonication time had a negative effect on the extraction of these components. This behavior may be explained by the balances in the extraction/degradation processes. Thus, it was recommended the use of high temperature, % ethanol in water and ultrasound time.

The influence of the parameters studied on the total antioxidant activity of the extracts by using DPPH was shown in Fig. 2-C. A direct influence of ultrasound power on the extraction was found and the use of low pH and high ultrasound power and temperature values was advisable. However, Bhuyan et al. [40] reported that power had no noteworthy consequences on the DPPH scavenging capacity.

Fig. 2-D showed the influence of ultrasound power and % ethanol in water on the total antioxidant activity evaluated using ABTS assay at different temperatures. At low temperature, the highest antioxidant activity was obtained with high ultrasound power and % ethanol in water. At medium–high temperatures, higher antioxidant activities were obtained and less significant influence of both parameters was revealed. Therefore, high temperature, ethanol-enriched solvents and low ultrasound power were recommended. However, Zhou et al. [43] reported denaturation of some thermo-sensitive non-phenolic antioxidants at a temperature above 60 °C.

From the results, a similar trend of the main variables involved in the antioxidant activity measured by DPPH and ABTS assays was observed [25,45].

3.3. Antioxidant activities of Eucalyptus globulus leaves extracts

Table 1 shows that the maximum extraction yields for each parameter were obtained using different values of pH, temperature, % ethanol in water and ultrasound time. The maximum TPC (114.6 mg gallic acid g^{-1} of dry leaves) was obtained using 15% EtOH at pH 5 and ultrasound power of 40 W at 50 °C during 10 min. This value was higher than those obtained using MAE (79.9 mg gallic acid g^{-1} of dry leaves) [41] and orbital shaker (88–93 mg gallic acid g^{-1} of dry leaves) [3,41], but lower than that obtained using soxhlet extraction (240 mg gallic acid g^{-1} of dry leaves) [11] and MeOH (148 mg gallic acid g^{-1} of dry leaves) [16].

Otherwise, maximum TFC (24.73 mg epicatechin g^{-1} of dry leaves) was obtained using 15% EtOH at pH 3 with ultrasound power of 40 W at 30 °C during 15 min. The best conditions for ABTS (107.49 mg ascorbic acid g^{-1} of dry leaves) were 25% EtOH at pH 5 and ultrasound power of 120 W at 30 °C during 5 min, whereas that for DDPH (107.19 mg ascorbic acid g^{-1} of dry leaves) were 30% EtOH at pH 0 and ultrasound power of 80 W at 60 °C during 10 min. Comparison with results for TFC, DPPH and ABTS antioxidant capacity in leaves of eucalyptus found in the literature was difficult because they were expressed in different units. Literature results for TFC are given in rutin equivalents [3,38] and we use epicatechin as the standard. ABTS and DPPH found in the literature are expressed as trolox equivalent [3,41] and we use ascorbic acid as the standard.

Therefore, four methods for the determination of the antioxidant capacity in extracts have been proposed. The optimal extraction parameters to obtain the highest content of bioactive compounds (TPC and TFC) and the highest antioxidant activities (ABTS and DPPH) were determined based on the values obtained by response surface methodology. From the statistical analysis of the obtained results for these analyses in the proposed experimental design, in which as a fundamental purpose, the conditions of maximum removal and minimum degradation of the studied compounds are considered, the optimum values of each parameter (T, pH, t, EW and P) have been calculated.

In summary, we selected some conditions of commitment consisting of high temperature (50 °C), low pH (3), medium operation time (10 min), high % ethanol in water (30%) and medium ultrasound power (60 W). The results obtained were 88.5 mg gallic acid g^{-1} of dry leaves for TPC; 11.1 mg epicatechin g^{-1} of dry leaves for TFC; 80.2 mg ascorbic acid g^{-1} of dry leaves for DPPH and 83.9 mg ascorbic acid g^{-1} of dry leaves for ABTS.

To evaluate ultrasounds effect, an experiment was performed under the same conditions of pH, temperature, % ethanol in water and extraction time, but without ultrasounds. The results shown extraction yields were lower in ultrasounds absence (51,1%, 40,9%, 78,8% and 78,9% for TPC, TFC, DPPH and ABTS, respectively).

Thus, industrialization of the proposed technique can be possible by experimental validation and scaling up of the lab parameters in terms of extraction time, yield, chemical composition and quality of environmentally friendly bioactive compounds to a pilot scale. Small scale-up of extraction techniques have been reported for MAE [46], UAE [47] and ASE techniques in the literature. Few published studies on the use of MAE [31,48], UAE [49] and ASE [49] on industrial processing of plant secondary metabolites have been found in the literature.

3.4. Chemical composition of Eucalyptus globulus leaves extract

The chemical composition and the relative content (%) of bioactive compounds (terpenes) in the optimized *Eucalyptus globulus* extract has been performed by GC–MS analysis (Table 3 and Fig. 3).

The chromatographic analysis showed the presence of 11 terpene compounds. The major compound was Eucalyptol with a relative content of 67.29%, followed by pinocarvone, pinocarveol and viridiflorol with 11.33%, 11.11% and 5.22%, respectively. The rest of the compounds found have a minority presence with respect to the previous ones. Similar results have been obtained by other authors [21,40,50–52].

4. Conclusions

Ultrasound extraction as a techno-economically suitable technique for the extraction of antioxidants in *Eucalyptus globulus* leaves should be considered. All the variables studied (pH, temperature, % ethanol in water, ultrasound power and ultrasound time) have a significant influence on the extraction of the compounds studied. The relative influence of these variables changes for the selected antioxidant activity assay. Although temperature is the most important variable in the extraction of most of the studied compounds. In addition, several terpenes (Eucayptol, camphenol, pinocarveol, pinocarvone, *trans*-p-mentha-1(7),8-dien-2-ol, *cis*-p-mentha-1(7),8-dien-2-ol, *exo*-2-Hydroxycineole, α-Terpineol acetate, alloaromadendrene, epiglobulol and viridiflorol) have been detected in extracts of *Eucalyptus globulus* leaves by GC–MS. The positive effect of ultrasonic temperature, time and power on extraction yield has been demonstrated. However, under high values of these parameters, a decrease in the extraction yield of selected compounds may occur. This decrease may be due to denaturation/degradation of the extracted compounds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2021.105654.

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