Optimization of solvent extraction of antioxidants from *Eucalyptus globulus* leaves by response surface methodology: characterization and assessment of their bioactive properties

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ABSTRACT

The extraction of antioxidants from plant biomass is of high interest and it requires processing conditions that preserve the bioactivity of these compounds. The optimization of the solvent extraction of antioxidants from the leaves of *Eucalyptus globulus* is a key point for its exploitation at industrial scale. In this work, the Box-Behnken experimental design was used to evaluate the effects of three independent variables (temperature, time and ratio of ethanol/water) on the response of extraction yield, total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity. Under optimal extraction conditions, the response values were 32.7% for extraction yield, 92.9 mg GAE/g dried leaf and 53.7 mg RE/g dried leaf for TPC and TFC, respectively, with antioxidant levels of 205.4, 363.4 and 185.2 mg TE/g dried leaf as determined by the DPPH, ABTS and FRAP methods, respectively. The extract obtained under optimal conditions was characterized by TGA, FTIR and Py-GC/MS. The Py-GC/MS analysis revealed that the main components found in the extract are sesquiterpenes, eudesmol, γ-eudesmol and globulol. Additionally, the antimicrobial activity of the extract was tested against several pathogens by showing capability to inhibit their growth. Therefore, the extract from *E. globulus* leaves could be used as a "natural" bioactive agent in several industrial applications.

Keywords:

Eucalyptus globulus leaf, Extraction optimization, Antioxidant activity, Antimicrobial activity, Characterization

1. INTRODUCTION

Eucalyptus (*Eucalyptus globulus* L.), a tall and strong tree from the family Myrtaceae, represents approx. a 27% of the total wood volume and it is one of the most important forest species in Galicia (Vázquez et al., 2008). The main use of the eucalyptus wood is the production of cellulose pulp followed by the manufacturing of boards and panels (Vázquez et al., 2008). Large amounts of wastes are generated during eucalyptus wood industrial processing, including bark, wood rejects, branches and leaves, which are mainly used for energy production or just left in the forest for soil amendment and fertilization purposes (Santos et al., 2012). In this perspective, the valorization of these residues would improve the industrial process both economically and environmentally. In fact, in the last years, the biomass wastes have attracted great interest as source of high-added-value compounds as a response of the depletion of fossil fuels, within the biomass refinery concept (Santos et al., 2012; Mota et al., 2012).

Some of the interesting compounds that can be obtained from the biomass leftovers belong to the group of those that present antioxidant activity; this group is receiving an increasing attention by the scientific community because they play an important role both in the maintaining of human health as in the preservation of foods (Paz et al., 2015; Harkat-Madouri et al., 2015). The antioxidant activity is usually attributed to the presence of phenolic compounds including hydroxybenzoic acids, hydroxycinnamic acids, flavonoids and tannins (Boulekbache-Makhlouf et al., 2012). In this regard, these compounds are responsible for their broad spectrum of bioactivities, including antioxidant, anticarcinogenic, antiallergenic, anti-inflammatory, and antimicrobial effects, among others (Santos et al., 2012; Harkat-Madouri et al., 2015); accordingly, they are extensively used in the food and pharmaceutical industry.

The increasing demand for natural antioxidants as alternative to less safe synthetic antioxidants has fostered intense research in this field (Boulekbache-Makhlouf et al., 2013). In this sense, in the last two decades, biomass wastes provide an attractive possibility as source of natural antioxidants because they are abundant and inexpensive (Fernández-Agulló et al., 2015; Mota et al., 2012). At this respect, many research works have demonstrated the high potential of

several eucalyptus by-products as natural sources of biologically active phenolic compounds (Santos et al., 2012). Boulekbache-Makhlouf et al. (2013) have reported antioxidant and antibacterial capacities of the phenolic extract of *E. globulus* fruits. Moreover, the bark also presents antioxidant (Vázquez et al., 2008) and anti-proliferative activities associated to the phenolic compounds present (Mota et al., 2012). Boulekbache-Makhlouf et al. (2012) have also reported the characterization of several polyphenolic constituents in extracts from leaves of this species. The essential oil from eucalyptus has been recently evaluated for its antibacterial properties against bacteria responsible of oral infectious diseases (Harkat-Madouri et al., 2015).

When it comes to ensure the efficient recovery of bioactive compounds from natural sources, extraction is a common approach. This stage must keep and extract most of the bioactive substances contained in a biomass residue and it must fulfil other requirements: versatility towards a wide range of compounds and conditions as well as a cost-effective and simple procedure (Fernández-Agulló et al., 2015; Díaz Reinoso et al., 2012; Thoo et al., 2013). Different factors can influence the extraction process efficiency, such as the type and concentration of solvent, extraction time or temperature (Fernández-Agulló et al., 2015). Commonly, bioactive compounds can be extracted by means of different organic solvents or a mixture of organic solvents and water (Díaz Reinoso et al., 2012; Mota et al., 2012). Among the available solvents, ethanol has been widely used because is one of the more environmentally friendly extracting agents and it is proclaimed as safe in accordance with the European Food Safety Authority (EFSA) and FAO/WHO Expert Committee on Food Additive (EFSA, 2011; Otero-Pareja et al., 2015). The available literature about the extraction of phenolic compounds from *E. globulus* leaves does not provide information regarding the combined effects of the main operational variables such as temperature, time, and solvent concentration on the extraction process. This information is crucial to expand the knowledge of the bioactivity of extracts from *E. globulus* leaves and it would contribute to use this material as a source of high-added-value compounds.

In this context, this work deals with the study of the influence of selected operational variables (temperature, time, and ethanol/water ratio) on the extraction of phenolic compounds with antioxidant activity from *E. globulus* leaves using the response surface methodology (RSM). The antioxidant activity was screened with three different methods (DPPH, ABTS and FRAP), and complemented with the measurements of total phenolic content (TPC) and total flavonoid content (TFC). In addition, the maximum extraction of phenolic compounds, flavonoids, and antioxidant activity was obtained by applying the optimal extraction conditions. These experimental values were compared with the ones predicted by the model. The extract obtained under optimal conditions was characterized by TGA, FTIR and Py-GC/MS for more in-depth knowledge about its composition. Finally, this extract was used to carry out the preliminary assessment of the antimicrobial activity against Gram positive and Gram negative bacteria. To our knowledge, this is the first time that the thermal stability and the determination of the types of functional groups as well as the evaluation of bioactive properties of this type of extracts are made.

2. MATERIALS AND METHODS

2.1. Materials

The *E. globulus* leaves were harvested in April, 2015 from cultivated plants located at Ourense, Spain. Samples were cleaned, air dried during 4 days to 9% average moisture, ground, sieved to pass 0.5 mm, and packed in sealed plastic bags and stored at -20 ºC until required. All the chemicals and reagents were of analytical grade. Ethanol, methanol, gallic acid, rutin, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu reagent, ABTS (2,2'-azino-di(3-ethylbenzothiazoline-6-suslfonic acid), TPTZ (2,4,6-tri(2-pyridyl)-S-triazine), DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium carbonate, sodium acetate 3-hydrate, potassium persulfate, acetic acid, hydrochloric acid, iron(III) chloride hexahydrate and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Barcelona, Spain). The Müeller Hinton (MH) broth and MH agar were purchased from Fluka (Barcelona, Spain).

2.2. Solid – liquid extraction

Eucalyptus leaves were extracted in an orbital shaker with temperature control (Adolf Kühner AG, Birsfelden, Switzerland) using aqueous ethanol as solvent. Two grams of dried leaf were placed in 100 mL Erlenmeyer flasks using a solid/liquid ratio fixed of 1:20 g/mL and a shaking speed of 120 rpm. The extracts were filtered through filter paper (Whatman Ashless, Grade 42) under vacuum and the filtrate was analyzed. The extracts for antibacterial activity were concentrated using a rotary evaporator (Buchi Rotavapor R-205, Flawil, Switzerland) at 35 ºC with reduced pressure (<100 mbar) to half of the initial volume. The concentrated extract was then freeze-dried and stored in glass bottles at -20 ºC until further utilization.

2.3. Experimental Design

The effects of the temperature (T_E, ºC), time (t_E, min), and ethanol concentration (%) were studied on the extraction yield and extracts properties. The selected independent variables and their variation ranges were determined based on preliminary studies (data not show) and other related research (Díaz Reinoso et al., 2012; Thoo et al., 2013). Response surface methodology (RSM) with a Box-Behnken design (BBD) with three replicates in the central point was used for the experimental design (Table 1) and optimization.

Experimental data were fitted using a second-order polynomial described by the equation 1:

$$y_j = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum \sum_{i<j=1}^{3} \beta_{ij} x_i x_j + \sum_{i=1}^{3} \beta_{ii} x_i^2 \qquad (1)$$

where y_j are the dependent variables (j = 1-6), β_0, β_i, β_{ij}, and β_{ii} are the regression coefficients calculated from the experimental results by the least-squares method, and x_i and x_j are the dimensionless, normalized independent variables, with variation ranges from -1 to 1. Experimental data were fitted using the regression analysis function of Microsoft Excel's Data

Analysis Add-In, USA. The adequacy of the model was determined by evaluating the lack of fit, the coefficient of determination (R^2) and the F-test value obtained from the analysis of variance.

2.4. Determination of the optimum conditions and validation of the model

A multi-response surface optimization was used to maximize the selected response variables at the same time. The selection criteria were based on extracts with high content of phenolic compounds, flavonoids and antioxidant activity (according to the values obtained by DPPH, ABTS and FRAP methods). The optimal extraction conditions were estimated using the desirability function of the software Statgraphics Centurion version XVI (Statpoint Technologies Inc., Warrenton, VA, USA). Model validation was carried out by performing the experiments at the optimal extraction conditions and a comparison between the values predicted by each model and the experimental data was made.

2.5. Characterization of the extracts

2.5.1. Total non-volatile solids

The content of non-volatile solids in extracts was measured by oven-drying at 105 ºC until constant weight and it is a measure of the extraction yield, calculated as the percentage weight of non-volatile solids per 100 g of dried leaf (EY, % w/w dried leaf). All samples were measured in triplicate.

2.5.2. Determination of total phenolic (TPC) and flavonoid content (TFC)

Total phenols content (TPC) was determined by the Folin-Ciocalteu method (Singleton & Rossi, 1965). Gallic acid was the reference standard and the results were expressed as mg of gallic acid equivalents (GAE)/g dried leaf. Total flavonoid content (TFC) was measured using the method described in Blasa et al. (2005). Rutin was used as standard and the results were expressed in mg of rutin equivalents (RE)/g dried leaf. Each assay was carried out in triplicate, and the mean value was calculated.

2.5.3. Determination of antioxidant capacity

2.5.3.1. α,α-Diphenyl-β-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was carried out according to the method described in Brand-Williams (1995) with slight modifications. Briefly, 2 mL of a 6 x 10^{-5} M methanolic solution of DPPH was added to 0.2 mL of an ethanolic solution of the extract. The decrease in absorbance at 515 nm was recorded after 16 min.

2.5.3.2. Trolox equivalent antioxidant capacity (TEAC)

TEAC was monitored using the ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay according to Thaipong et al. (2006). The ABTS radical cation ($ABTS^{\bullet+}$) was obtained by the reaction of 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration). The mixture was maintained at room temperature and protected from light for 12-16 h before use. Next step consisted on diluting the $ABTS^{\bullet+}$ solution with phosphate buffer saline (PBS, pH 7.4) to an absorbance of 0.70 at 734 nm and was carried to 25 ºC. Six minutes after the addition of 2 mL of diluted $ABTS^{\bullet+}$ solution to 20 µL of diluted extracts, the decrease in the absorbance was recorded.

2.5.3.3. Ferric Reducing Antioxidant Power (FRAP)

This assay was performed according to the procedure described by Thaipong et al. (2006) with some modifications. In short, the reagent was made by mixing 25 mL of 300 mM acetate buffer (pH 3.6) and 2.5 mL of a 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl and 2.5 mL of a 20 mM $FeCl_3 \cdot 6H_2O$ solution. The diluted extracts (0.1 mL) were mixed with 3 mL of the FRAP reagent. The absorbance was recorded after 6 min at 593 nm.

For all three methods, trolox was used as standard and results were expressed in mg of trolox equivalents (TE)/g dried leaf as mean of three replicates.

2.5.4. Thermogravimetric analysis (TGA)

Thermogravimetric analysis of the extract from eucalyptus leaves obtained under optimal conditions of extraction was carried out using a TGA/SDTA RSI analyzer 851 Mettler

Toledo. Between 3 and 5 mg of extract were tested under a nitrogen atmosphere at a heating rate of 10 ºC/min from 25 ºC to 950 ºC, in order to study the mass loss characteristics of the thermal decomposition process of extract.

2.5.5. Fourier transform infrared spectroscopy (FTIR)

The FTIR analysis of the eucalyptus extract obtained at the optimal extraction conditions was carried out on a Perkin Elmer Spectrum Two FT-IR spectrometer. A total of 8 scans were accumulated in transmission mode with a resolution of 4 cm^{-1}. The spectrum was recorded from a range of 4000 to 600 cm^{-1}.

2.5.6. Pyrolysis-Gas chromatography-Mass spectrometry analysis (Py-GC/MS)

The extract from eucalyptus leaves obtained at the optimal conditions of extraction was analyzed by Py-GC/MS using a 5150 Pyroprobe pyrolyzer (CDS Analytical Inc., Oxford, PA) connected to an Agilent 6890 gas chromatograph with an Agilent 5973 (Agilent Technologies In., USA) mass spectrometer. The GC was equipped with a 30 m x 0.25 mm x 0.25µm film thickness HP-5MS ((5%phenyl)-methylpolysiloxane) column and used helium as the carrier gas. The pyrolysis was performed at 600 ºC for 15 s at 20 ºC/ms, being the interface kept at 260 ºC. The GC oven was programmed from 50 ºC (2 min) to 120 ºC (5 min) at 10 ºC/min, then to 280 ºC (8 min) at 10 ºC/min and finally to 300 ºC (10 min) at 10 ºC/min. The compounds were identified by comparing their mass spectra with those from the National Institute of Standards Library (NIST), as well as with those compounds reported in the literature (Fernández-Rodríguez et al., 2017). Peak molar areas were calculated for the products with peak area ratio higher than 0.4%, and the summed identified peak areas were normalized to 100% in order to calculate the relative abundance of the identified compounds (Chen et al., 2015).

2.6. Antimicrobial activity

2.6.1. Microorganisms and culture conditions

The antibacterial properties of the extracts were tested against the following bacterial strains: *Listeria innocua* (NCTC 10528), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Bacillus cereus* (DSM 4313), *Pseudomonas aeruginosa* (10145) and *Salmonella enteritidis* (ATCC 3076). Stock cultures were maintained in cryovials with glycerol at 15% (v/v) and maintained at -80 ºC before use. The strains for experiments were grown in sterile Mueller-Hinton broth (MHB) at 37 ºC for 8-12 h.

2.6.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of eucalyptus leaf extracts

Antimicrobial activity of eucalyptus extracts was determined according to the Clinical and Laboratory Standards Institute guidelines described in the document M07-A9 with the modifications proposed by Paz et al. (2015). The MIC and the MBC determination were performed by a serial dilution technique, using sterile 96-well microtiter plates. Each well was filled with a total volume of 280 µL containing ca. 10^6 colony forming units (CFU)/mL of test pathogenic bacteria, fresh MHB and diluted extract sample in dimethyl sulfoxide (DMSO). The tested extract concentrations ranged from 10 to 75 mg/mL. A positive control was prepared with each microorganism and MHB supplemented with DMSO (at the highest concentration used in the experiments carried out with the extracts); negative control contained MHB with extract sample without inoculum. The microplates were incubated for 24 h at 37 ºC.

The minimal inhibitory concentration (MIC) value was defined as the lowest concentration of extract capable of inhibiting microbial growth after 24 h of incubation at 37 ºC. The minimal bactericidal concentration (MBC) values were taken as the lowest concentration of extract that killed more than 99.9% of the bacteria after 24 h of incubation at 37 ºC.

Bacterial growth reductions in the samples with extract respect to positive control were evaluated by comparing viable cell counts at 0 h and after the incubation period. For this, serial decimal dilutions of each culture were placed on MH agar, and visible bacterial colonies were counted after incubation at 37 ºC for approximately 48 h. All assays were performed in triplicate to confirm the reproducibility of the results.

3. RESULTS AND DISCUSSION

3.1. Optimization of the extraction conditions

In order to obtain the best active extracts, the joint optimization of the most influential variables was addressed using BBD combined with RSM. **Table 1** summarizes the experimental plan, including the fixed variables and their values, the independent variables (temperature, time and ethanol concentration) and their variation ranges as well as the dependent variables (extraction yield, TPC, TFC, DPPH, ABTS and FRAP). In this work, ethanol was chosen due to practical aspects as cost, non-toxicity and extracting capacity (Díaz Reinoso et al., 2012).

Table 2 shows the set of experiments carried out that corresponded to a BBD, as well as the experimental results determined for the dependent variables. **Table 3** shows the regression coefficients obtained for each model according to a second-degree polynomial, their statistical significance (based on a Student's t test), the parameters measuring the correlation (R^2) and statistical significance (Fisher's F test) of the models. The value determined for R^2 for all variables was higher than 0.96, which indicates that the model is adequate to represent the real relationships among the selected variables. Moreover, the high values of F confirm the good fit of data. The *p* value for each equation term was calculated to assess the contribution of linear interaction, and quadratic effects of the independent variables.

Using the calculated significant regression coefficients, at the 90% confidential level, six quadratic regression equations for the EY (y_1), TPC (y_2), TFC (y_3), DPPH (y_4), ABTS (y_5) and FRAP (y_6) were set up:

$$EY = 27.92+1.47x_1+2.27x_2+1.75x_3+1.52x_2x_3+1.34x^2_1 \quad (2)$$

$$TPC = 89.36+3.53x_1-1.77x_3+6.84x_2x_3+2.79x^2_1-10.21x^2_3 \quad (3)$$

$$TFC = 53.4+1.06x_1+1.67x_3+1.18x_1x_3+2.02x_2x_3-1.32x^2_1-1.61x^2_2-4.46x^2_3 \quad (4)$$

$$DPPH = 196.56+4.87x_1-9.66x_3+8.47x_1x_2+5.63x_1x_3+10.91x_2x_3-29.48x^2_3 \quad (5)$$

$$ABTS = 352.76+18.45x_1+6.21x_2-15.34x_3+15.66x_2x_3-8.88x^2_1-19.78x^2_2-36.12x^2_3 \quad (6)$$

$$FRAP = 189.88-10.31x_3+4.85x_1x_3+11.80x_2x_3-18.87x^2_3 \quad (7)$$

3.1.1. Extraction yield (EY)

The extraction yields of the experiments were in the interval of 24.5 to 33.1% (**Table 2**). The minimum and maximum values corresponded to the experiments 1 and 11, respectively. The extraction yield achieved in this study was higher than the one obtained in related works as described by Pereira et al. (2014) who attained a 27% using 70% methanol for the extraction of *E. globulus* leaves.

In accordance with the regression coefficients (see **Table 3**), all of the independent variables influenced significantly the extraction yield (y_1), and the interaction between the extraction time and % EtOH had also a significant effect on variable y_1, as well as the quadratic effect of temperature. The combined effect of the temperature with the extraction time or with the ethanol concentration was not significant, as can be seen in **Table 3**.

The interaction effects between the temperature and the ethanol concentration in the extraction yield (EY) for a set value of extraction time (x_2=1) is showed in the **Figure 1a**. In this surface plot, it can be observed that an increase on the temperature and ethanol concentration involved an increase on the extraction yield. However, extraction yield was more influenced by the ethanol concentration than by the temperature. This behavior is due to the high influence of the quadratic term of temperature on the extraction yield equation. The maximum extraction yield predicted by the model was 35.7%, which was achieved at the highest temperature (50 ºC), time (300 min) and ethanol concentration (80%). This result is in agreement with the data reported by Liyana-Pathirana and Shahidi (2005) which evidenced that the extraction process is favoured by the higher temperature, contact time and ethanol concentrations since the solubility of compounds, the diffusion of the solvent and the weakening of the plant tissues are enhanced.

3.1.2. Total phenolic content (TPC)

The TPC was significantly affected by the temperature and the ethanol concentration as evidenced by the values of the coefficients concerning variable y_2 (**Table 3**). The interaction

between extraction time (x_2) and ethanol concentration (x_3) was also significant within a 99% confidence interval.

Figure 1b shows the response surface of TPC in function of temperature and ethanol concentration while extraction time was held constant at 165 min ($x_2 = 0$). As can be observed, the TPC was positively influenced in all of its variation range by the temperature. In contrast, we observed that the ethanol concentration had two different effects on TPC: in the first half of its variation range this parameter had a positive effect on TPC; however, after the optimum level was attained, the increase in the ethanol concentration had a negative effect on TPC. This behaviour is accounted in the model by the negative contribution of the quadratic coefficient of the ethanol concentration in the regression equation (see **Table 3**). These results can be explained in basis to the influence of the polarity of solvents and compounds on the extraction of phenolic compounds. The use of a single solvent might not be effective for the extraction of bioactive compounds however, a mixture of alcohol with water is more effective in phenolic compounds extraction than alcohol alone (Wang et al., 2013; Rajha et al., 2014). This fact suggests that most of the bioactive compounds present in *E. globulus* leaf extracts have polar nature.

The highest value of TPC (95.8 mg GAE/g dried leaf) was attained at 50 ºC, EtOH of 45% and 123 min. The results obtained in our study compared favourably with those published by Piwowarska and González-Alvarez (2012), where the TPC from forest biomass increased when the alcohol concentration increased up to approximately 50%. Mota et al. (2012) also reported a maximum of phenolic compounds from *E. globulus* bark with 50% of EtOH. A similar trend has been observed for the ultrasonic-assisted extraction of antioxidant compounds from blackberry leaves (Aybastıer et al., 2013).

3.1.3. Total flavonoid content (TFC)

Taking into account the values of the model coefficients collected in **Table 3**, it can be inferred that the two independent variables (x_1, x_3) and the three quadratic terms (x^2_1, x^2_2 and x^2_3) significantly influenced the TFC, although these latter showed a negative effect on y_3. The

TFC was positively affected by the interaction effects between the extraction temperature and ethanol proportion as well as extraction time and ethanol concentration. Among the significant terms, ethanol concentration had the strongest effect on the extraction of flavonoids.

Figure 1c shows the response surface for TFC in function of extraction temperature and ethanol concentration for a fixed value of extraction time (x_2= 0). Similar to the response of TPC, TFC increases when the ethanol concentration and temperature increases, but this increase occurred only up to 60% ethanol and 44 ºC, respectively. Increments in these two parameters above these values caused a decline on TFC that reaffirmed the negative quadratic effect of both regression model coefficients (see **Table 3**). It must be considered that this influence was more accused for the increment of ethanol concentration than for temperature (the ethanol concentration has largest negative quadratic coefficient). The highest TFC predicted by the model was 54 mg RE/g dried leaf and it was obtained at 60% EtOH, 44 ºC and 207 min. These results compared favourably with the reported by Sheng et al. Li (2013) that obtained similar values of TFC of extract from *Flos populi* using an ethanol concentration of 45.92%, 95 ºC and 147 min.

TFC results were in line with previous studies, where the flavonoids compounds extraction from plant matrices were more effective using a two-solvent system instead of an individual solvent system (Zhang et al., 2011; Rajha et al., 2014). Similar findings were also observed for the extraction of flavonoids from natural sources (Liyana-Pathirana and Shahidi, 2005; Sheng et al., 2013; Thoo et al., 2013). Regarding optimal extraction temperature, the result obtained in this study was lower than the reported by others authors (Sheng et al., 2013; Rajha et al., 2014). In this sense, Liyana-Pathirana and Shahidi (2005) informed that high temperatures may facilitate bioactive molecules extraction while promoting the degradation of those which were solubilized at low temperatures. Thus, extraction conditions using low temperatures may be interesting for extracting flavonoids, which are heat-sensitive compounds.

3.1.4. Antioxidant Capacity

The influence of extraction conditions on the antioxidant capacity of *E. globulus* leaf extracts was determined using three different antioxidant assays: DPPH, ABTS and FRAP. ABTS and DPPH assays are based on the antioxidant's ability to react with or neutralize free radicals generated in the assay systems, whereas the FRAP assay measures the reduction of Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron) in the presence of antioxidants.

Taking into account the values of the regression coefficients calculated for DPPH (**Table 3**), it can be inferred that the most influential variable on the DPPH was ethanol concentration, which showed negative effect in linear and quadratic terms. The temperature and interaction between all the independent variables had a positive effect on the DPPH.

With respect to the antioxidant capacity by ABTS method, the three independent variables studied (temperature, time and ethanol concentration) and the three quadratic terms significantly affected the ABTS value, as well as the interaction between extraction time and ethanol concentration. From the results of FRAP antioxidant activity (**Table 3**), it can be deduced that the variation pattern was similar to the one observed for DPPH and ABTS values, being the ethanol concentration the most influential variable.

Figure 1 (d, e, f) shows the effects of extraction temperature and ethanol concentration on antioxidant capacity measured by the assays of DPPH, ABTS and FRAP for a fixed extraction time (165 min). In general, an increase in extraction temperature at a fixed ethanol value provoked an increase in DPPH and ABTS values. This dependence of antioxidant capacity with extraction temperature was in accordance to the previous studies reported by other authors (Thoo et al., 2013; Aybastıer et al., 2013). On the other hand, the antioxidant activity measured by FRAP assay was not influence by the extraction temperature.

Figure 1 (**d, e, f**) represents the influence of ethanol concentration on the antioxidant capacity; it can be noted that all the antioxidant capacity values increased with the ethanol concentration up to approximately 50% and then began to decrease. Regarding to the model equation, this trend is described by the negative sign of linear and quadratic coefficients of the ethanol concentration. Similar results for the dependence of antioxidant capacity (analyzed by the FRAP, DPPH and ABTS methods) on solvent concentration were obtained by Piwowarska

and González-Alvárez (2012) for forestry biomass extracts. Aybastıer et al. (2013) also reported the antioxidant capacity by ABTS and CUPRAC method decreases after reaching a methanol concentration of 50%. In contrast, Thoo et al. (2013) reported that ABTS and DPPH values for extracts from *Morinda citrifolia* increased with an increase in ethanol concentration. Different types of bioactive extracted compounds depending on the ethanol concentration can explain this behaviour (Thoo et al., 2013).

The highest trolox equivalents in the DPPH assay (206 mg TE/g dried leaf) was obtained when the extraction was carried out at 50 ºC, ethanol concentration of 53% and 300 min. Regarding maximum content of TE in ABTS assay (363.9 mg TE/g dried leaf), it was achieved at the following operating conditions: 50 ºC, ethanol concentration of 44% and 174 min. The maximum value of antioxidant capacity measured by the FRAP method (196.7 mg TE/g dried leaf) was predicted for conditions defined by the extraction temperature of 25 ºC, ethanol concentration of 29% and 35 min.

The TPC, TFC and antioxidant capacity from *E. globulus* leaves were influenced significantly by the ethanol concentration as shown the response surface plots of all the dependent variables studied.

Due to the existing relationship between content of the antioxidant compounds and their bioactive properties as well as a potential synergistic effect among the diverse molecules present in the extract (El Darra et al., 2012; Rajha et al., 2014), a simultaneous optimization was carried out to maximize the content of phenolic compounds, flavonoids and their antioxidant activity.

3.1.5. Optimization of extraction conditions and validation of the model

The aim of the optimization was to determine the extraction conditions that would provide simultaneously the greatest content of phenolic compounds, flavonoids, and antioxidant activities. The extraction yield was not considered in the optimization phase, since in the optimum conditions of this variable the content of TPC, TFC and DPPH, ABTS and FRAP decreased drastically. Statgraphics Centurion XV software was used to carry out the optimization. For this purpose, the values of responses of each variable were converted using a

desirability function. This function was considered to disclose the combination of the extraction variables evaluated capable of maximizing the selected responses (TPC, TFC, DPPH, ABTS and FRAP) at the same time.

The optimum conditions for the independent variables were: 50 ºC, 225 min and ethanol concentration of 56%. Higher temperatures (60 and 70 ºC) were also evaluated, and although TPC and TFC were kept constant, the antioxidant activity decreased (data not shown). **Table 4** shows the predicted and experimental results for all variables selected in this study. The suitability of the response surface methodology model for quantitative predictions was verified by the satisfactory agreement between the predicted and measured values. These findings also justified the selection of the Box-Behnken design, which was demonstrated to be accurate and reliable for predicting the total phenolic content, flavonoids and the antioxidant capacities of ethanolic extracts from eucalyptus leaves.

Under these optimum conditions, the extraction yield value was 32.7%, TPC and TFC were of 92.7 mg GAE/g dried leaf and 53.7 mg RE/g dried leaf, respectively, with DPPH of 205.4 mg TE/g dried leaf, ABTS of 363.4 mg TE/g dried leaf and FRAP of 185.2 mg TE/g dried leaf. These values were similar to those found optimal when using the single objective functions (95.8 mg GAE/g dried leaf, 54 53.7 mg RE/g dried leaf, 205.8 mg TE/g dried leaf, 363.9 mg TE/g dried leaf, 196.6 mg TE/g dried leaf).

3.2. Thermal stability of the extract

The knowledge of thermal transitions of extract from eucalyptus leaves is essential to elucidate the inter-relation between its chemical structure and its degradation and this would allow to predict the yield of pyrolysis products (Liu et al., 2016). The literature collects thermogravimetric studies that have shown that the thermal stability of several antioxidants decreases with the increase of temperature (Cordeiro et al., 2013). The eucalyptus leaves extract has potential applications as source of natural antioxidants for vegetable oils subjected to high temperatures and also, it could be incorporated into thermoplastic films, in which the thermal stability is a parameter to be taken into account (Cordeiro et al., 2013). For this purpose, a

thermogravimetric analysis was carried out to determine the thermal stability of the extract obtained at the optimal conditions. **Figure 2** (**a, b**) shows the thermogravimetry (TG) and first derivate (DTG, decomposition rate) curves.

Below 105 ºC, the extract from eucalyptus leaves showed a mass loss of 1.65% associated to the gradual evaporation of the moisture. The extract exhibited a high thermal stability, maintaining its mass almost unaltered up to 150 ºC (weight retention of 96.5%, mass loss of only 3.5%) as the TG/DTG curves represent (**Figure 2a, 2b**). The major decomposition took place between 195 and 482 ºC; in this interval the greater mass loss occurred (49.3%). Within this second step, we can distinguish several decomposition stages that overlap and that can be attributed to the degradation of compounds groups with different structural features present in the extract (**Figure 2b**). Three peak temperatures were identified: 195, 310 and 482 ºC. The mass loss in this range of temperatures may also be attributed to the volatilization/decomposition of bioactive constituents, possibly from terpene derivatives. This behavior is in agreement with the reported by Cordeiro et al. (2013) for rosemary extract leaves. Finally, the third step, in the range from 482 to 947 ºC, with a mass loss of 20.6% can be related to antioxidants with complex structures. The extract presented a 19.65% of the char residue obtained at 947 ºC corresponding to non pyrolyzable compounds. The ash content of the extract was 4.76%.

3.3. Pyrolysis-GC/MS

Due to the complexity of the structure of the compounds present in the extracts of eucalyptus leaves, the results obtained by gas chromatography (data not shown) were not conclusive, so it was decided to make pyrolysis-GC/MS to identify some compounds present in extracts. The compounds detected by pyrolysis-GC/MS are presumably part of complex structures present in the extracts.

The volatile components generated in the pyrolysis of the extract obtained at the optimal extraction conditions were identified based on the total ion chromatogram of each peak and by literature (Nunes et al., 2010). **Table 5** shows the identified constituents on extract from *E.*

globulus leaves. Pyrolysys-GC/MS is an analytical technique commonly used for identifying components of complex polymeric materials, without requiring previous preparation steps of the sample. However, it has also been used to analyze extracts of plants on organic solvents, as applied in this study. For example, Py-GC/MS was used to analyze the top value-added biomedical and bioactive constituents of benzene/ethanol extracts of oil-tea cake (Liu et al., 2010) and bamboo root (Lv et al., 2010). In another study, this technique was used to evaluate the identity and reproducibility of different brands and batches of commercially available samples of *Cymbopogon citratus Stapf* (sold as tea) by analysing its hexane extract (Oliveira et al., 2010).

In total, 21 compounds were identified in the extract from the eucalyptus leaves. The sesquiterpenes were the most abundant terpene derivatives achieving a 24.34% on the crude extract (Table 5). Of these, the major compounds identified in the crude extract of eucalyptus leaves were β-eudesmol (12.84%), γ-eudesmol (3.36%) and globulol (2.87%) that belong to the oxygenated sesquiterpene group and alloaromadendrene (3.80%) which is a sesquiterpene hydrocarbon. The presence of α-selinene and α-gurgujene was detected in low concentrations. The detected compounds in this work have been found in oils from leaves of other species of *Eucalyptus* such as *E. gilli* (Hassine et al., 2012) *inter alia*. Moreover, some compounds identified in the extract of eucalyptus (β-eudesmol and γ-eudesmol) have been described in other plants such as *Anaxagorea brevipes* (de Alencar et al., 2016).

The detection of guaiacol and syringol can be indicative of the presence of small fragments of lignin on the extract of *Eucalyptus* leaves because they are the most common aromatic structures of lignin. Compounds such as *o*-cymene, catechol and phenol detected on the extract have been found on lignin-derived monomeric products (Sasaki and Goto, 2008).

3.4. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the extract obtained from eucalyptus leaves under the optimal conditions of extraction is illustrated in the **Figure 3**.

As seen in Figure 3, the strong absorption bands at 3308 cm^{-1} corresponds to the joint contribution of the OH stretching vibrations coming from different chemical environments, which is characteristic of polyphenolic extracts (Mohan Kumar et al., 2013). The bands at 2942 cm^{-1} may correspond to CH and CH_2 stretching vibration of aliphatic hydrocarbons, and at 1710 cm^{-1} which belong to carbonyl groups from dimerized saturated aliphatic acids (Vázquez et al., 2008). The FTIR spectrum of eucalyptus leaf extract shows bands at 1611 cm^{-1} due to C = C aromatic ring stretching vibration, bands at 1445 cm^{-1} resembles C–C stretch (in-ring) to aromatic, 1311 cm^{-1} for C-N stretching vibration for aromatic amines and 1035 cm^{-1} for C-N stretching vibration of aliphatic amines that are attributed to the bands assigned to phenolic compounds (e.g. flavonoids and polyphenols) (Wang et al., 2014). The band at 1185 cm^{-1} can be assigned to C–O stretching, possibly for presence of esters from hydrolysable tannins (Grasel et al., 2016).

These bands sustain the presence of phytochemical constituents in eucalyptus leaf extracts that include functional groups such as phenols, amines, carboxyl and carbonyl.

3.5. MIC and MBC determination

The antibacterial activity of eucalyptus leaf extracts was assessed against three Gram positive (*S. aureus*, *L. innocua* and *B. cereus*) and three Gram negative (*E. coli*, *P. aeruginosa* and *Salmonella* sp.) bacterial strains. In order to determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) for each microorganism (**Table 6**) different concentrations of the extract obtained in the optimal conditions were tested.

The results presented in **Table 6** allow us to infer that the eucalyptus leaf extracts inhibited the growth of all the microorganisms tested, suggesting the presence of different bioactive molecules in their composition that may have additive or synergistic effects. Regarding to the MIC and MBC results obtained, it can be deduced that Gram-positive bacteria were more susceptible to the eucalyptus extracts than the Gram-negative bacteria. The greater susceptibility of Gram-positive bacteria to antibacterial agents is in line with previously reported results for several plants extracts and may be justified by the different membrane

structure (Paz et al., 2015; Harkat-Madouri et al., 2015). The Gram-negative bacteria have a lipopolysaccharide outer membrane, which provides greater protection to the cell by avoiding the interaction with the extracts (Harkat-Madouri et al., 2015; Paz et al., 2015). In this sense, it has been reported that the antimicrobial activity of phenolic compounds and flavonoids is due to their interaction with the cellular membrane constituents causing perforation and/or a reduction in membrane fluidity (Tsuchiya and Iinuma, 2000).

The MIC values obtained for the microorganisms tested were in the range 30-45 mg/mL. The results showed that the bacterial strains *L. innocua* and *S. aureus* were the most sensitive ones for crude leaf extracts of *E. globulus* with a MIC value of 30 and 35 mg/mL respectively; while higher resistance was shown by *P. aeruginosa* and *Salmonella* sp with a MIC value of 45 mg/mL. In general, the MIC values reported here were significantly higher than the results reported by other authors for extracts from eucalyptus leaves. For instance, Pereira et al. (2014) informed MIC values in the range from 0.625-2.5 mg/mL for eucalyptus leaf extracts against *P. aeruginosa*. Moreover, Luís et al. (2014) reported MIC values of 5 and 2.5 mg/mL against *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, respectively, using alcoholic extracts of *E. globulus* stump wood. In contrast, MIC analysis for other leaves plant extracts (rich in phenolic compounds) showed considerably higher values. Indeed, Piccirillo et al. (2013) reported a MIC value of 100 mg/mL using leaf ethanolic extracts from ginja cherry plant against *P. aeruginosa*, *E. coli* and *Salmonella*.

The MBC values were evaluated between 35-50 mg/mL (**Table 6**) and showed an analogous trend than MIC values. It is important to highlight that the differences found in the antimicrobial activity of extracts reported by different studies could be explained by various factors such as the differences in the purity of the extracts, in the phenolic content and in the specific composition of bioactive molecules, as well as it could be attributed to the different extraction methodologies employed, the antimicrobial assays selected and the strain specific resistances (Boulekbache-Makhlouf et al., 2013; Pereira et al., 2014; Fernández-Agulló et al., 2015).

Nowadays, the increasing resistance to conventional antimicrobial agents, such as antibiotics against human pathogens, makes necessary an intense research and development of new antimicrobial substances from natural sources. In this aspect, extracts from *E. globulus* leaves have great potential because of their various bioactive compounds and biological properties, such as antioxidant and antimicrobial properties (Pereira et al., 2014; Boulekbache-Makhlouf et al., 2012).

4. CONCLUSIONS

The optimization of the extraction conditions of bioactive compounds from *E. globulus* leaves was successfully carried out using the Box-Behnken design. The results showed that ethanol concentration had the greatest impact on the TPC, TFC and antioxidant capacity (measured by DPPH, ABTS and FRAP). The optimal extraction conditions were: temperature of 50 ºC, time of 225 min, and ethanol concentration of 56%. The predicted values of optimum extraction parameters were well consistent with the experimental values. The thermogravimetric analysis showed that the extract from eucalyptus leaves is stable at high temperatures evidencing the presence of bioactive compounds with complex structures. The chemical composition from Py-GC/MS allowed to identify compounds that belong mostly to the sesquiterpene group as well as lignin derivatives. The FTIR analysis evidenced the presence of phytochemical constituents in eucalyptus leaf extracts.

The extracts produced using those conditions displayed strong antioxidant activity, as well as antimicrobial activity against Gram-positive and Gram-negative bacteria. The results obtained in this work showed the potential of the leaves from *E. globulus* as an economical source of antioxidant and antimicrobial agents, with prospective use in different applications.

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Notes

The authors declare no competing financial interest.

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FIGURE CAPTIONS

Figure 1. Response surface for extraction yield (a); TPC (b); TFC (c); ABTS (d); DPPH (e) and FRAP (f) in function of temperature and % EtOH.

Figure 2. Thermogravimetric analysis spectra of antioxidant extract from eucalyptus leaves (a). Derivative DTG curves of antioxidant extract from eucalyptus leaves (b).

Figure 3. FTIR spectrum of extract from eucalyptus leaves

Table 1. Experimental variables involved in the study

Variable	Definition and units	Nomenclature	Value or range
Fixed	Liquid to solid ratio of extraction (v/w)	LSR	20 mL/g
	Shaking speed (rpm)		120
Independent	Extraction temperature (ºC)	T_E	25-50 ºC
	Extraction time (min)	t_E	30-300 min
	Ethanol concentration (% v/v)	EtOH	20-80 %
Dependent	Extraction yield (%)	EY or y_1	
	Total phenolic content (mg GAE/g dried leaf)	TPC or y_2	
	Total flavonoid content (mg RE/g dried leaf)	TFC or y_3	
	DPPH* radical scavenging activity (mg TE/g dried leaf)	DPPH or y_4	
	ABTS cation radical scavenging activity (mg TE/g dried leaf)	ABTS or y_5	
	Ferric reducing antioxidant power (mg TE/g dried leaf)	FRAP or y_6	

Table 2. Operational conditions assayed (expressed in terms of dimensional and dimensionless independent variables) and experimental results obtained for dependent variables y_1 to y_6

Experiment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Independent Variables															
T_E (ºC) or x_1	25 (-1)	25 (-1)	25 (-1)	25 (-1)	37.5 (0)	37.5 (0)	37.5 (0)	37.5 (0)	37.5 (0)	37.5 (0)	37.5 (0)	50 (1)	50 (1)	50 (1)	50 (1)
t_E (min) or x_2	30 (-1)	165 (0)	165 (0)	300 (1)	30 (-1)	30 (-1)	165 (0)	165 (0)	165 (0)	300 (1)	300 (1)	30 (-1)	165 (0)	165 (0)	300 (1)
EtOH (%) or x_3	50 (0)	20 (-1)	80 (1)	50 (0)	20 (-1)	80 (1)	50 (0)	50 (0)	50 (0)	20 (-1)	80 (1)	50 (0)	20 (-1)	80 (1)	50 (0)
Dependent Variables															
EY or y_1 (%)	24.5	26.4	29.5	30.2	25.3	26.1	28.0	27.7	28.1	26.2	33.1	28.0	29.4	32.5	32.5
TPC or y_2 (mg GAE/g dried leaf)	88.1	81.0	73.9	87.0	83.5	69.1	88.7	89.5	89.9	71.4	84.5	93.5	89.3	83.6	91.8
TFC or y_3 (mg RE/g dried leaf)	48.9	46.5	46.4	50.2	46.0	46.4	53.8	53.5	52.9	44.2	52.7	51.8	46.5	51.1	51.0
DPPH or y_4 (mg TE/g dried leaf)	203.2	178.8	144.2	178.6	183.8	146.6	194.8	197.6	197.3	160.2	166.8	193.4	179.9	167.9	202.6
ABTS or y_5 (mg TE/g dried leaf)	305.1	306.2	265.3	313.9	315.2	261.0	351.6	350.9	355.9	301.3	309.8	335.7	348.2	312.1	341.8
FRAP or y_6 (mg TE/g dried leaf)	190.4	182.3	152.6	185.3	189.1	144.2	188.6	190.6	190.5	168.5	170.9	185.1	179.9	169.6	188.1

Table 3. Regression coefficients and statistical parameters measuring the correlation and significance of the models

Coefficient	y_1	y_2	y_3	y_4	y_5	y_6
b_0	27.92[a]	89.36[a]	53.4[a]	196.56[a]	352.76[a]	189.88[a]
b_1	1.47[a]	3.53[a]	1.06[b]	4.87[b]	18.45[a]	1.53
b_2	2.27[a]	0.07	0.62	-2.35	6.21[c]	0.50
b_3	1.75[a]	-1.77[c]	1.67[a]	-9.66[a]	-15.34[a]	-10.31[a]
b_{12}	-0.30	-0.14	-0.55	8.47[a]	-0.71	2.01
b_{13}	0.02	0.35	1.18[c]	5.63[b]	1.25	4.85[b]
b_{23}	1.52[a]	6.84[a]	2.02[b]	10.91[a]	15.66[a]	11.80[a]
b_{11}	1.34[a]	2.79[c]	-1.32[c]	0.60	-8.88[c]	0.14
b_{22}	-0.45	-2.04	-1.61[b]	-2.74	-19.78[a]	-2.82
b_{33}	0.21	-10.21[a]	-4.46[a]	-29.48[a]	-36.12[a]	-18.87[a]
R^2	0.989	0.962	0.965	0.986	0.975	0.983
F-exp	53.69	14.11	15.45	40.89	21.89	32.97
Significance level (%)	99.980	99.527	99.613	99.999	99.831	99.936

[a] Significant coefficients at the 99% confidence level.
[b] Significant coefficients at the 95% confidence level.
[c] Significant coefficients at the 90% confidence level.

Table 4. Predicted and experimental values under optimum conditions based on multiple response of TPC, TFC, DPPH, ABTS and FRAP (extraction temperature = 50 ºC, extraction time = 225 min, ethanol concentration = 56%)

	Response					
	Extraction yield (%)	TPC (mg GAE/g dried leaf)	TFC (mg RE/g dried leaf)	DPPH (mg TE/g dried leaf)	ABTS (mg TE/g dried leaf)	FRAP (mg TE/g dried leaf)
Predicted value	32.3	95.5	53.44	204	357.7	189
Experimental value[a]	32.7 ± 1.2	92.9 ± 1.00	53.7 ± 1.42	205.4 ± 4.6	363.4 ± 3.7	185.2 ± 1.8

Mean ± standar deviation (SD) of three determinations (n=3) from three extract replications

Table 5. Chemical composition Pyrolysis-GC/MS of the extract obtained from eucalyptus leaves in the optimal conditions extraction

Compound	Retention time (min)	Molecular formula	Characteristic ions (m/z)	Relative content (g compound/100g extract)
Toluene	3.9	C_6H_5-CH_3	91,92,65	0.42
Furfural	4.8	$C_5H_4O_2$	96,95,67	0.60
5-methyl furfural	7.0	$C_6H_6O_2$	110,109,53	0.65
Phenol	7.3	C_6H_6O	94,66,65	0.99
o-cymene	8.0	$C_{10}H_{14}$	119,134,91	0.50
Guaiacol	9.1	$C_7H_8O_2$	109,124,81	0.79
2,4-dimethyl phenol	10.4	$C_8H_{10}O$	107,122,77	0.71
Catechol	11.1	$C_6H_6O_2$	110,64,63	1.48
3-methoxy catechol	12.9	$C_7H_8O_2$	140,125,97	0.60
4-methyl catechol	13.7	$C_7H_8O_2$	124,123,78	0.46
Syringol	15.6	$C_7H_8O_2$	154,139,111	0.96
Vanillin	16.9	$C_8H_8O_3$	151,152,81	0.91
δ-guaiene	17.3	$C_{15}H_{24}$	107,79,81	0.89
Alloaomadedrene	17.8	$C_{15}H_{24}$	68,67,79	3.80
α-selinene	18.9	$C_{15}H_{24}$	189,107,93	1.19
α-gurgujene	19.1	$C_{15}H_{24}$	161,105,204	1.28
Globulol	20.4	$C_{15}H_{26}O$	149,146,177	2.87
γ-eudesmol	21.1	$C_{15}H_{26}O$	161,189,204	3.36
β-eudesmol	21.4	$C_{15}H_{26}O$	159,145,202	12.84
Tetradecanoic acid	22.6	$C_{14}H_{28}O_2$	73,60,129	1.54
n-Hexadecanoic acid	24.8	$C_{16}H_{32}O_2$	73,60,57	4.75

Table 6. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) (mg/mL) of extracts from *Eucalyptus globulus* leaves. All assays were done in triplicate

Microorganism	**MIC**	**MBC**
E. coli	40	50
P. aeruginosa	45	50
Salmonella sp	45	50
S. aureus	35	40
L. innocua	30	35
B. cereus	40	45

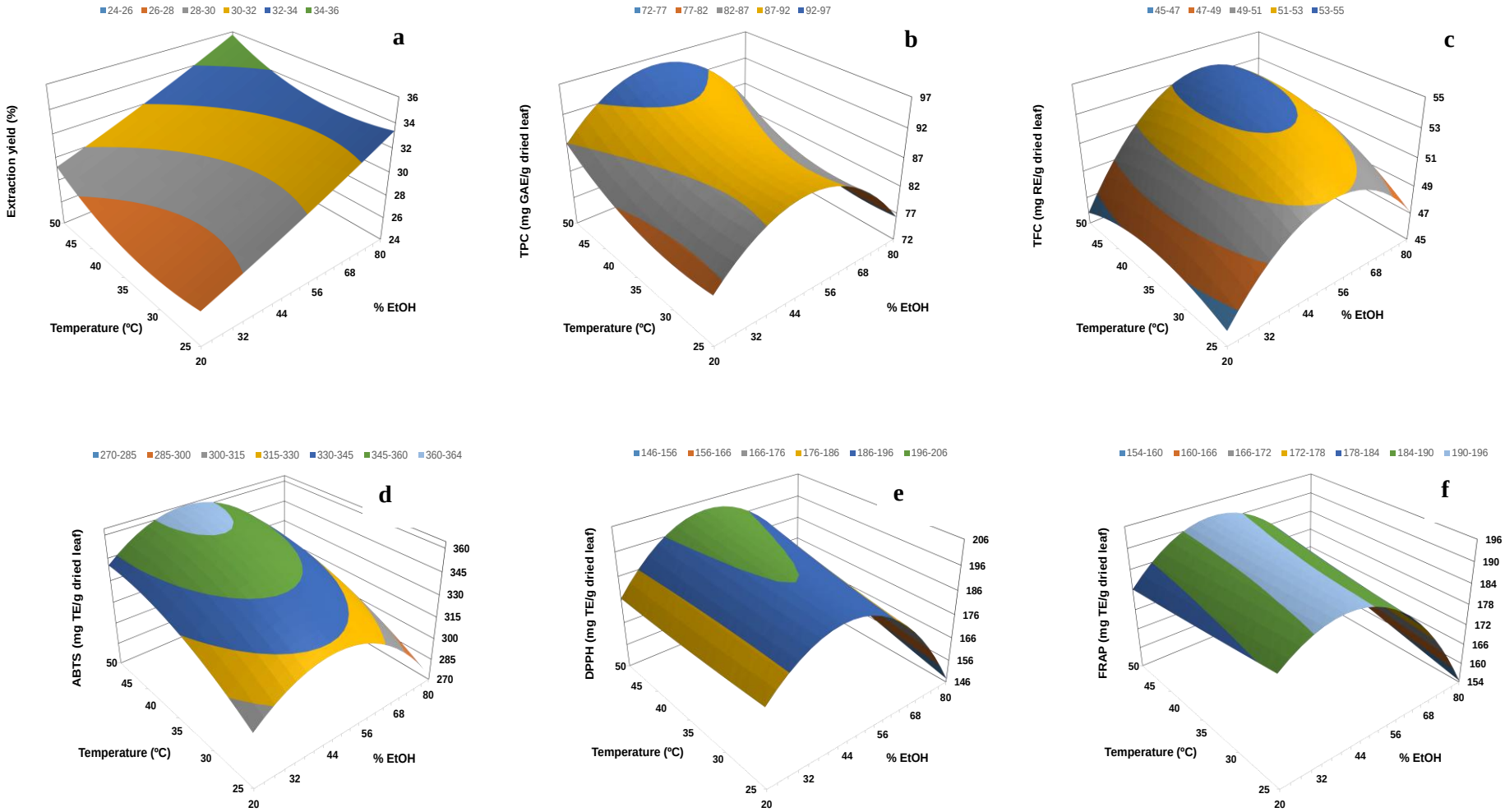
24-26
26-28
28-30
30-32
32-34
34-36
a
Extraction yield (%)
Temperature (ºC)
% EtOH
72-77
77-82
82-87
87-92
92-97
b
TPC (mg GAE/g dried leaf)
Temperature (ºC)
% EtOH
45-47
47-49
49-51
51-53
53-55
c
TFC (mg RE/g dried leaf)
Temperature (ºC)
% EtOH
270-285
285-300
300-315
315-330
330-345
345-360
360-364
d
ABTS (mg TE/g dried leaf)
Temperature (ºC)
% EtOH
146-156
156-166
166-176
176-186
186-196
196-206
e
DPPH (mg TE/g dried leaf)
Temperature (ºC)
% EtOH
154-160
160-166
166-172
172-178
178-184
184-190
190-196
f
FRAP (mg TE/g dried leaf)
Temperature (ºC)
% EtOH

Figure 1

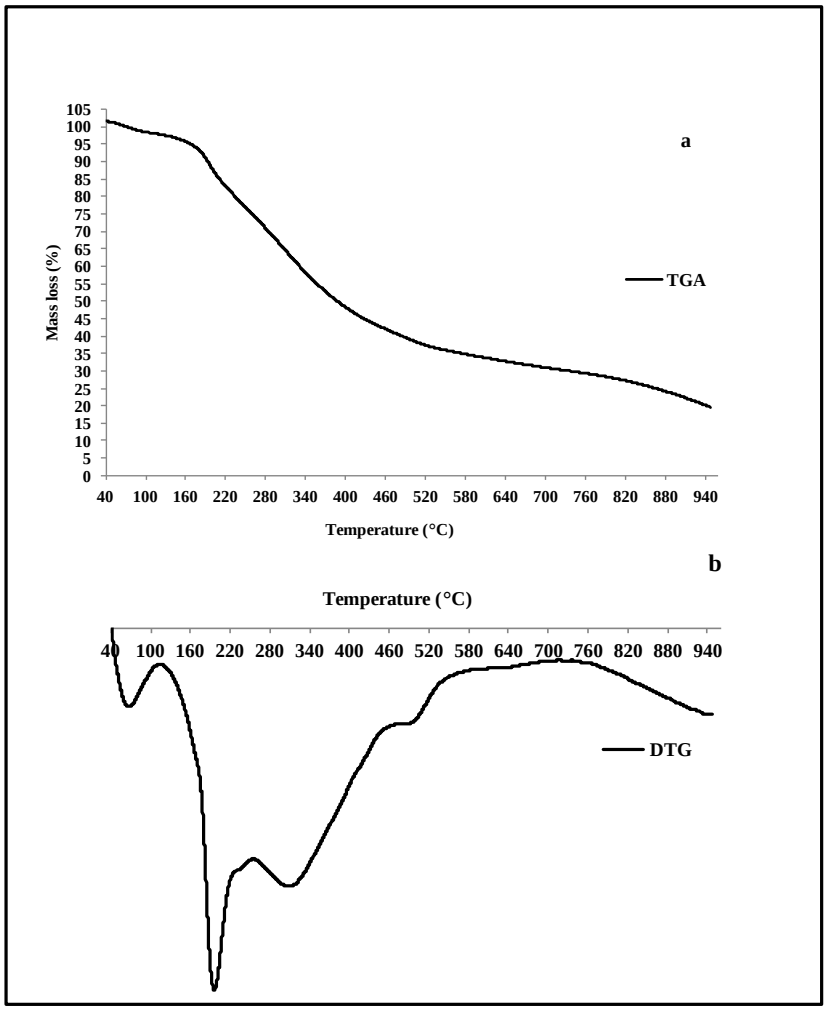
a
Mass loss (%)
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
TGA
40 100 160 220 280 340 400 460 520 580 640 700 760 820 880 940
Temperature (°C)
b
Temperature (°C)
40 100 160 220 280 340 400 460 520 580 640 700 760 820 880 940
DTG

Figure 2

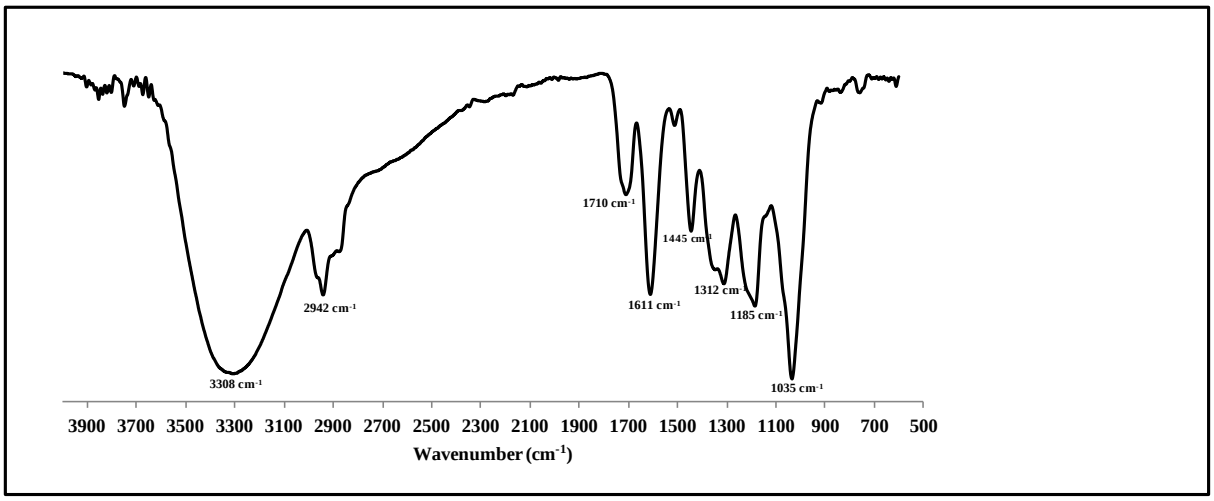
3308 cm-1
2942 cm-1
1710 cm-1
1611 cm-1
1445 cm-1
1312 cm-1
1185 cm-1
1035 cm-1
3900 3700 3500 3300 3100 2900 2700 2500 2300 2100 1900 1700 1500 1300 1100 900 700 500
Wavenumber (cm-1)

Figure 3