

# EUCALYPTUS GLOBULUS BARK AS A SOURCE OF POLYPHENOLIC COMPOUNDS WITH BIOLOGICAL ACTIVITY

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## ABSTRACT

*Eucalyptus globulus* bark, one of the main by-products of the pulp and paper industry in Southern Europe, is a potential source of valuable chemicals. In this work, chemical composition of *E. globulus* bark was studied with detail, including its carbohydrate composition. Response surface methodology (RSM) modeling and optimization was developed for the selective extraction of polyphenolic material from *E. globulus* bark. The RSM method was based on the Box-Behnken design, aiming to obtain the optimal combination of extraction conditions considering the parameters ethanol percentage in the extraction medium, temperature and time. Conditions for maximum of polyphenols in the extract are 52% ethanol, extraction temperature of 82.5°C and extraction time of 264 min. The polyphenolic compounds - quantified as gallic acid equivalents - in the extract produced at the optimal conditions was 32%, corresponding to about 2% of bark weight, with a carbohydrate co-extraction of about 1.6% of bark carbohydrate content. Some of the extracts revealed low values of IC<sub>50</sub> against human breast cancer cells, indicative of high biological activity. This work has demonstrated the potential of *E. globulus* bark as a source of polyphenolic compounds with anti-proliferative activity and gives a positive contribution to the increase in products portfolio diversification in pulp industry and biorefineries.

**Keywords:** Bark, bioactivity, carbohydrates, extraction optimization, phenolic compounds

## INTRODUCTION

Bark of *E. globulus* is classified as by-product and it is currently used as fuel to suppress power needs at mill site. Within the

biorefinery concept applied to pulp and paper industry, the possibility of bark exploitation as raw material for high-added value applications has fostered in the last years the research on its potential for several applications. *E. Globulus* bark contains considerable quantities of polyphenolic material (Conde *et al.*, 1996; Vázquez *et al.*, 2008). This is of great importance for the industry, since the extracts of this byproduct find increasing applications as active substances for cosmetics, food additives and even in pharmaceutical products. However, from the industrial point of view, the economic sustainability of the extraction process claims for process efficiency. To achieve this, the study of the optimum extraction conditions is a step forward. The modeling and optimization of extraction processes leads to efficient and economical designs of important industrial operation. The response surface methodology (RSM) is an effective modeling tool that can simulate and optimize complex processes through an easier arrangement of the variables and interpretation of results comparatively to exhaustive and time-consuming conventional methods. It has been widely employed for optimizing the extraction of high added-value components, including phenolic compounds from several sources (Yang *et al.*, 2009; Zhang *et al.*, 2010). The aim of this work is to develop an extraction process that favors the withdrawal of phenolic compounds from *E. globulus* bark applying the RSM concepts. For this, three important factors - extraction time, extraction temperature and ethanol percentage -, were analyzed using Box-Behnken design. Additionally, by the evaluation of biological activity in a critical health field, our research intends to open the perspective for a new and valuable utilization for bark chemical components.

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## MATERIALS AND METHODS

### Characterization of bark

Bark of *E. globulus* was collected at a bleached pulp Portuguese mill. Tappi standard methods were used for bark analysis (T 204 om-88, T 207 om-99, T 212 om-02, T 222 om-02, T 211 om-93). For monosaccharide quantification, acid hydrolysis and acid methanolysis were applied. The products of acid hydrolysis were quantified by anion-exchange chromatography using a DX500 Dionex chromatograph equipped with a CarboPac PA1 column (4 x 250 mm) and pre-column PA1 (4 x 50 mm). Detection was performed with a ED50 Electrochemical Detector Dionex operating in pulse amperometric mode. The eluent was 2 mM NaOH (1 mL/min), and the cleaning eluent (applied after each run) was 0.3M NaOH. For quantification, external calibration was used and fucose was the internal standard. The products of acid methanolysis (24 h, 100°C, HCl 2M in anhydrous methanol) were trimethylsilylated and analyzed by GC-MS and GC-FID, allowing the quantification of the neutral and acid monosaccharides as described (Costa *et al.*, 2012). External calibration was performed with monosaccharide standards with reference to sorbitol as internal standard; glucuronic acid was the standard for 4-*O*-methylglucuronic acid. Nitrobenzene oxidation was performed as described in literature using about 100 mg of milled bark (Pinto *et al.*, 2010).

### Extracts characterization and definition of the dependent variables

Extraction yield is expressed as weigh of total nonvolatile solids (quantified by TAPPI T652m-89) per 100 g of dried bark (% bark wt.). Total phenolic compounds were quantified by Folin-Ciocalteu method as described in literature (Cadahía *et al.*, 1997) and expressed as g of gallic acid equivalent per 100 g of extract (TPC, % w/w<sub>extract</sub>). The carbohydrates in extracts were accessed by acid methanolysis as described for bark characterization. Total carbohydrates value was calculated as the sum of all monosaccharide converted to homopolysaccharide and expressed as g of total carbohydrate per 100 g of dried extract (TC, % w/w<sub>extract</sub>). The analyses were done in duplicate and the mean value was calculated.

### Design of experiments and statistical analysis

The process variables selected to this study were:  $X_1$  - time of extraction (30-360 min),  $X_2$  - temperature (25-140°C) and  $X_3$  - solvent composition given by the ethanol percentage in water (0%-80%) aiming to assess their isolated effect and interaction effects on extract composition. Therefore, bark extraction experiments were planned according to Box-Behnken design with three central point replicates, maintaining the liquid:solid ratio of 8. The extractions were performed in M/K digesters with time control and liquid recirculation using 500 g of bark for

4 L of extractive medium. All experiments were performed in randomized order to minimize the effects of uncontrolled factors that may introduce a bias on the measurements. The extracts were collected, cooled and a fraction was freeze-dried. The remaining was stored in the freeze at  $N_2$  atmosphere. Response surfaces were built using the Unscrambler® software, with adjustment of the experimental results to a linear model with the integration of significant quadratic and interaction effects. Statistical significance was determined to 95% confidence interval. Three experimental responses are presented in this work:  $Y_1$  - extraction yield, % wt;  $Y_2$  - total phenolic compounds (TPC), %w/w<sub>extract</sub>;  $Y_3$  - total carbohydrate (TC), %w/w<sub>extract</sub>. A classical second-degree model was postulated for each experimental response  $Y_n$ , as follows:

$$Y_n = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j + \sum_{i=1}^3 \beta_{ii} X_i^2 + \varepsilon \quad (1)$$

where  $\beta_0$  is the model constant,  $\beta_i$  represent the principal effects associated with each independent variable and  $\beta_{ij}$  and  $\beta_{ii}$  represent, respectively, the model coefficients for interaction and quadratic effects of the independent variables (cross effects between variables) and  $X_i$  and  $X_j$  are the independent coded variables between -1 (lower limit) and 1 (upper limit);  $\varepsilon$  is the experimental error.

The validation of the models was accomplished by comparing the experimental and predicted values in each model for the optimal extraction conditions selected.

### Biological activity

Human breast cancer cells (obtained from American Type Culture Collection, Manassas, Va, USA) were incubated in an atmosphere of 95% air and 5%  $CO_2$  at 37°C (C150, Binder GmbH, Tuttlingen, Germany). The extracts were dissolved in cell culture medium or in dimethyl sulfoxide to a final concentration below 1%; controls received DMSO only. Cells were seeded in 96-well plates at  $2 \times 10^5$  cells/mL and, after 24 h, cells were incubated with the extracts at various concentrations. Cell viability was estimated by [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] (MTT) assay (CALBIOCHEM) as previously described (Mosmann, 1983). The results were expressed as percentage of cell viability relative to a control (cells without any test compound).  $IC_{50}$ , defined as the extract concentration necessary to cause 50% inhibition of cell viability, was calculated plotting the percentage of cell viability compared to a control (no added extract) against the different extracts concentration tested. All tests were performed at least in triplicate ( $n=3$ ) and the data are expressed as means  $\pm$  standard deviation. One-way analysis of variance (ANOVA) was used to test for differences between extracts activity.

## RESULTS AND DISCUSSION

### Chemical analysis of *E. globulus* bark

The chemical composition of bark is depicted in **Table 1**. Sequential extraction revealed higher content of lipophilic and ethanol/toluene extractives comparatively to values reported for *E. globulus* wood (Freire *et al.*, 2002), as well as higher content of polar material extracted with water and alkaline solutions (Pereira, 1988). Indeed, these are the main differences between bark and wood: the values found in hot water and 1% NaOH are 8.1% and 24.8%, respectively, while for wood 3.7% and 16.5% has been reported (Conde *et al.*, 1995). The higher values of solubility are attributed to the extraction of polyphenolic compounds, including low molecular weight lignin. In addition, the extracted material should also contain a fraction of soluble carbohydrates and calcium oxalates, which are abundant in bark of *E. globulus* (Quilhó *et al.*, 1999). The content of inorganics is within literature data (Pereira, 1988).

The value found for total lignin content (19.3%) is within the range found by other authors (18.6%-23.0%) (Pereira 1988). The analysis of bark lignin by nitrobenzene oxidation revealed a ratio of syringyl/guaiacyl units of 80/20, the same ratio reported for wood lignin; moreover the yield obtained suggests that the content of condensed structures of bark's lignin is similar to that of wood. (Pinto *et al.*, 2005).

Concerning carbohydrates in bark, the value obtained for neutral anhydro monosaccharides (66.5%) are higher than the value reported in literature (Vázquez *et al.*, 2008) (62.5%) and equivalent to the sum of pentosans and cellulose (67.0%), the average value obtained from bark with different origins published elsewhere (Pereira 1988). As far as is concerned, no data have been reported for uronic acids in bark of *E. globulus*.

**Table 1.** General composition of *E. globulus* bark

Parameter	% wt, dry weight
Ashes	2.3
Ethanol/toluene extractives	2.2
Dichloromethane extractives	0.71
Methanol extractives	2.8
<b>Solubility:</b>	
Cold water	2.4
Hot water	8.1
Aqueous solution 1% NaOH	24.8
<b>Lignin:</b>	
Klason	18.0
Acid soluble	1.4
<b>Carbohydrates:</b>	
Acidic anhydro monosaccharides	5.5
Neutral anhydro monosaccharides	66.5

**Table 2.** Neutral and acidic monosaccharides liberated by acid hydrolysis and acid methanolysis

Monosaccharide	% wt. - dried bark	
	Acid hydrolysis	Methanolysis
Ara	1.6	2.1
Xyl	10.4	11.8
Rha	(a)	0.36
Glc	49.7	2.3
Man	(a)	0.42
Gal	1.6	2.1
GalA	-	3.2
MeGlcA	-	2.3

(a) not detected

Carbohydrates were characterized employing two distinct methodologies: acid hydrolysis and acid methanolysis. The results, depicted on **Table 2**, showed glucose (Glc) as the main residue, followed by xylose (Xyl) and minor amounts of arabinose (Ara) and galactose (Gal).

Acid hydrolysis is the conventional method for polysaccharide composition analysis; however, labile uronic acids are degraded. Acid methanolysis was applied to achieve an efficient cleavage of the glycosidic linkage between neutral monosaccharides and uronic acids with no degradation, allowing the reliable quantification of both in the bark. Acid methanolysis was performed for different reaction times, and the highest yield for bark was achieved for 24 h; therefore, these data was considered for the calculation of carbohydrate composition in bark. In spite of all the advantages of acid methanolysis, the complete cleavage of cellulose is not achieved; therefore, for the accurate quantification of glucose in bark, the results from acid hydrolysis was considered; acid methanolysis was used for non-cellulosic polysaccharides quantification.

Cellulose content of *E. globulus* bark, given by Glc content (as homopolysaccharide) is about 50%. As wood, bark certainly contains also a significant fraction of hemicelluloses composed by Xyl, 4-*O*-methylglucuronic acid (MeGlcA) and Gal (glucuronoxylans) (Shatalov *et al.*, 1999), accounting for about 16% of bark weight. Small amounts of Ara, rhamnose (Rha) and mannose (Man) were also detected by acid methanolysis. Galacturonic acid (GalA), referred as constituent of galacturonans, was quantified in 3.2 wt.%. The Glc, quantified by methanolysis, could be attributed to a fraction of amorphous cellulose, glucomanans or even non-cellulosic glucans. Hence, this was not included in total sum, since all the Glc present is probably included in the value of cellulose (quantified by acid hydrolysis).

**Table 3.** Experimental values obtained for the different responses studied according to the Box-Behnken design. Predicted values are presented in parentheses

Conditions (Independent variables)				Dependent variables		
Run	(t, min)	(T, °C)	(Et, %)	Extract yield %w/w <sub>bark</sub>	TPC %w/w <sub>extract</sub>	TC %w/w <sub>extract</sub>
I	30	25	40	2.8 (3.6)	12.2 (12.0)	22.3 (20.4)
II	360	25	40	3.2 (1.7)	30.8 (27.3)	22.5 (23.0)
III	30	140	40	8.3 (8.8)	23.9 (27.4)	35.6 (36.6)
IV	360	140	40	19.4 (17.6)	20.7 (20.9)	38.9 (39.2)
V	30	82.5	0	4.4 (4.8)	20.0 (14.4)	26.7 (29.0)
VI	360	82.5	0	5.7 (8.3)	17.2 (18.8)	35.4 (31.6)
VII	30	82.5	80	3.2 (1.7)	21.0 (23.3)	20.5 (16.5)
VIII	360	82.5	80	4.3 (5.1)	25.9 (27.7)	18.7 (19.1)
IX	195	25	0	2.3 (1.7)	10.3 (10.3)	22.4 (22.5)
X	195	140	0	17.7 (17.3)	10.9 (14.9)	50.9 (49.7)
XI	195	25	80	2.3 (3.6)	15.5 (19.2)	19.7 (20.9)
XII	195	140	80	7.6 (9.1)	31.4 (23.7)	26.3 (26.2)
C(a)	195	82.5	40	5.7 (5.0)	31.1 (31.1)	22.3 (24.0)

(a) – average of the three replicates of the center point (runs XIII, XIV, XV)

### Optimization of extraction parameters

**Table 3** summarizes the experimental design matrix and the corresponding experimental values, resulting from the 15 experiments carried out. The three replicates (run XIII-XV) at the center of the design (C, in Table 3) were used for estimating the pure error.

The aim of optimization is to find the best combination of time of extraction, temperature of extraction and ethanol percentage, to maximize the TPC in the extract.

Through multiple regression analysis on the experimental data, predicted response  $Y$  for the extraction yield, total phenolic compounds (TPC<sub>extract</sub>) and total carbohydrates (TC<sub>extract</sub>) in the extract can be expressed by the following second-order polynomial equations in term of coded values:

$$\text{Extraction yield} = 4.99 + 1.73X_1 + 5.28X_2 - 1.59X_3 + 2.67X_1X_2 - 2.50X_2X_3 + 2.94X_2^2 \quad (2)$$

$$\text{TPC}_{\text{extract}} = 31.14 + 2.19X_1 + 2.28X_2 + 4.42X_3 - 5.46X_1X_2 - 2.63X_1^2 - 6.62X_2^2 - 7.47X_3^2 \quad (3)$$

$$\text{TC}_{\text{extract}} = 24.05 + 1.31X_1 + 8.10X_2 - 6.27X_3 - 5.50X_2X_3 + 5.78X_2^2 \quad (4)$$

where  $X_1$ ,  $X_2$  and  $X_3$  are the coded variables for extraction time and extraction temperature and ethanol percentage, respectively.

The statistical parameters obtained from the ANOVA are given in

**Table 4.** Statistical analysis of the fitted quadratic polynomial models

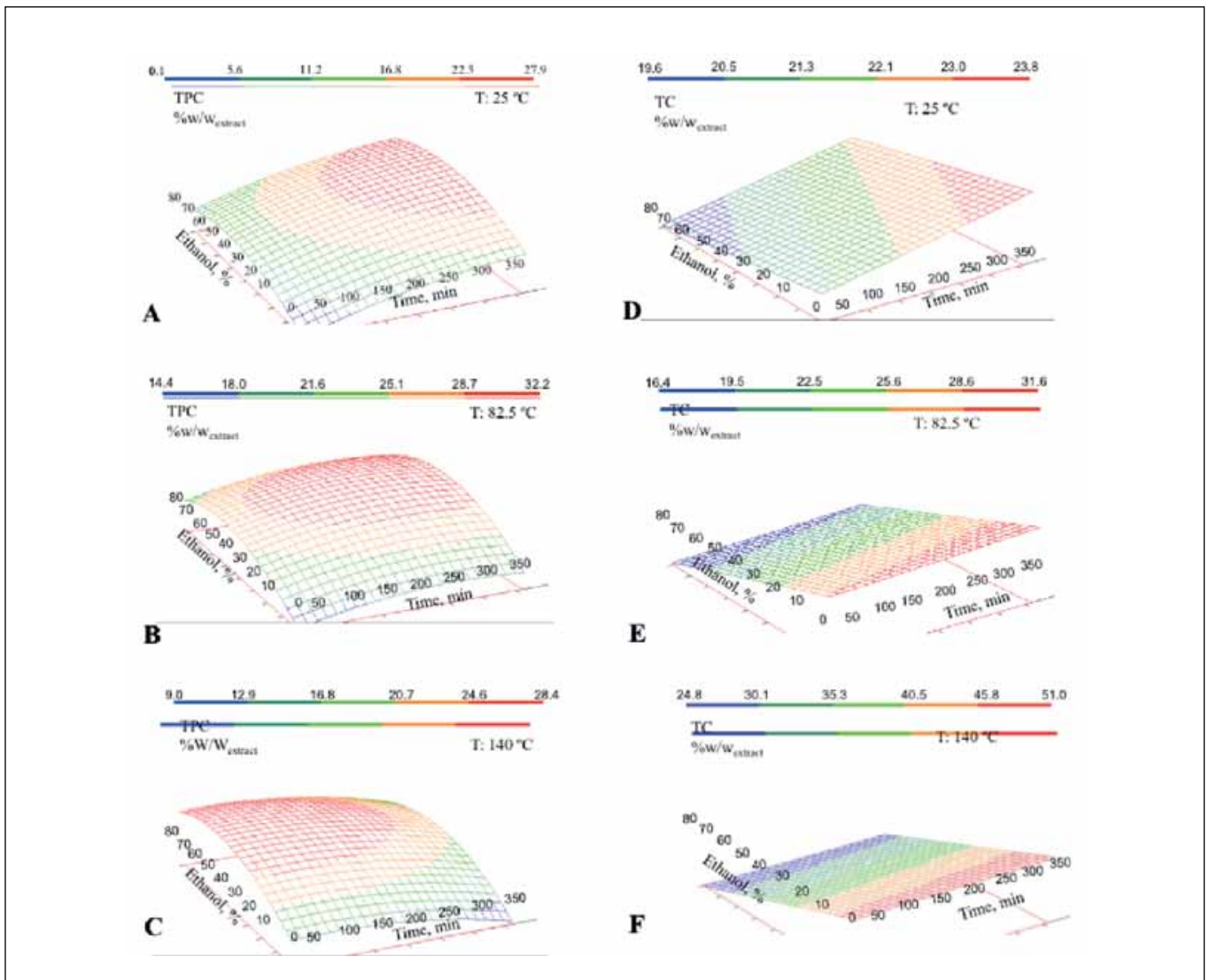
	Parameter	Extract yield (% wt.)	TPC (% w/w <sub>extract</sub> )	TC (%w/w <sub>extract</sub> )
p-value	Model	0.0002	0.0353	<0.0001
	Lack of fit	0.0015	0.0983	0.7228
	R2	0.941	0.814	0.935

**Table 4.** R<sup>2</sup> gives an indication of the total variability around the mean explained by the regression model.

The regression equations showed a good adjustment of the sample data since all R<sup>2</sup> were higher than 0.8.

p-Value inferior to 0.05 or 0.01 gives the significance of the model considering a confidence interval of 95% or 99%, respectively. p-Value for lack of fit (assuming a confidence interval of 95%) was not significant for TPC and for TC, but not so good for total extraction yield. Although not shown, the p-value for each equation term was also calculated to examine the contribution of linear, interaction and quadratic effects in the independent variables. Results had demonstrated that the temperature and time of extraction have a significant linear effect on extraction yield, while for the extraction of TPC the quadratics terms of temperature and ethanol concentration were very significant.

The relationship between independent and dependent variables is represented by 3D response surfaces (**Figure 1**) generated by the models. For each plot, temperature is kept constant and the responses were generated as a function of the time and % of ethanol in order



**Figure 1.** Three-dimensional response plots for TPC ( $Y_2$ ) and TC ( $Y_3$ ) as a function of ethanol % and time for (A, D) 25°C, (B, E) 82.5°C and (C, F) 140°C

to simplify visualization and interpretation. The interaction between ethanol concentration ( $X_3$ ) and extraction time ( $X_1$ ) for 25°C, 82.5°C and 140°C is shown on plots A, B and C for TPC, and D, E and F for TC.

From plots A to C it is possible to observe that a minimum of 40% ethanol is necessary to achieve the maximum for TPC in the extract, regardless the time and temperature of extraction. This is a necessary condition for definitely boost the selectivity of extraction for TPC. The increase of temperature led to the change of the TPC maximum to lower extraction times, clearly visible from plots A to C. The increase of temperature from 82.5°C to 140°C has as consequence the decrease of the required time to achieve the maximum. However, the TPC values obtained at 140°C are lower than for moderated temperature. Indeed, it is well know that the increase of temperature in the extraction process would accelerate the degradation of phenolic compounds, some of them bioactive. Carbohydrates extraction is promoted by low ethanol %, as

expected by the lower polarity of ethanol comparatively to water. As the ethanol percentage increases, the content of TC in the extract decreases. So, higher ethanol percentages in the extract medium, as well as low temperature, are not favorable to carbohydrates extraction. The effect of extraction time is attenuated with the increase of temperature, as demonstrated by plots D to F. Indeed, at 140°C, the variable time seems to be not relevant. This observation means that, at this temperature, the 'extractable' carbohydrates are most likely solubilized in the beginning of extraction, which is consistent with the polar nature of this family of compounds, and also with the already stated opposite effect on phenolic compounds. It is Interesting to note that even at low temperature, high ethanol % and low extraction time, about 16%-19% of the extract is composed by carbohydrates. This is probably due to the simultaneously high accessibility and availability in bark comparatively to phenolic compounds. Consequently, this would be always a contaminant in



**Table 5.** Predicted and experimental values of the response at the optimum conditions (264 min, 82.5°C, 52% ethanol)

Value	Extract yield (% wt.)	TPC (% w/w <sub>extract</sub> )	TC (%w/w <sub>extract</sub> )
Predicted	5.2	32.3	22.7
Experimental	5.2 (±0.2)	30.4 (±2.2)	21.2 (±0.9)

polyphenolic-rich extracts. This is the reason why the study of the conditions that promote the maximum of polyphenolic compounds in the extract - in detriment of other components -, is necessary if the perspective of bark valorization stated in this work is considered. Lower contaminants in the extract would certainly be an advantage in a downstream separation process.

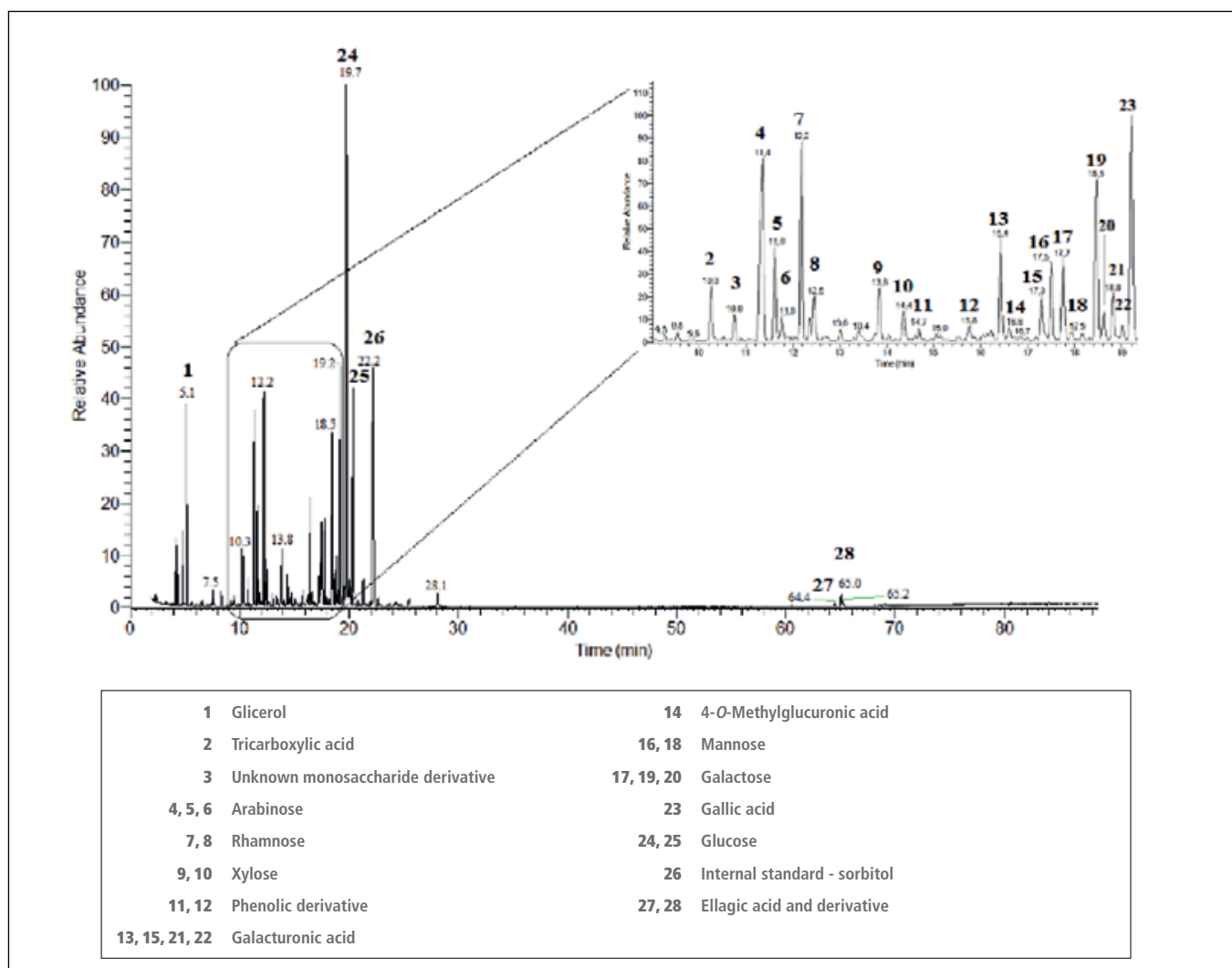
In this study, the aim of optimization was to find the conditions which gave the maximum of TPC in the extract. The extraction conditions predicted by the model are: 264 min of extraction time, 82.5°C of extraction temperature and 52% of ethanol percentage. Predicted and experimental results are depicted in **Table 5**.

The value found for TPC in the extract is very close to the experimental value; the difference is within the standard deviation of the quantification method. This indicates that the optimization achieved in the present study was reliable. At the optimum conditions, it is possible to obtain a polyphenolic-enriched extract containing about 32% of TPC quantified as gallic acid equivalents, and about 23% of carbohydrates.

Considering the important contribution of carbohydrates in the extracts, their content in the extracts was quantified based in the sum of individual residues released by acid methanolysis. A typical chromatogram is shown in **Figure 2** for the extract obtained at optimum conditions, assigning the peaks to the corresponding residue isomer.

Methanolysis of individual sugar residue leads to the formation of several products owing to the anomerization and ring isomerization processes.

Between 2 and 4 peaks are obtained for each monosaccharide converted to their corresponding TMS methyl glycoside derivative.



**Figure 2.** GC-MS chromatogram of the TMS methyl glycosides of monosaccharides obtained by acid methanolysis (2M HCl/MeOH, 4 hours of reaction, 100°C) of the extract obtained at optimized conditions

**Table 6.** Regulating activity of *E. globulus* bark extracts on cell proliferation as revealed by IC<sub>50</sub>

Extract	Extraction conditions			IC <sub>50</sub> (µg/mL)
	Time (min)	Temperature (°C)	Ethanol (%)	
I	30	25	40	No effect
IV	360	140	40	266.7±18.7
VIII	360	82.5	80	91.9±9.0
IX	195	25	0	No effect
X	195	140	0	No effect
XI	195	25	80	244.8±28.3
XII	195	140	80	220.0±19.6
Optimal conditions	264	82.5	52	176.4±15.7

These multiple peaks correspond to the  $\alpha$  and  $\beta$ -anomers and the pyranose and furanose ring forms of the monosaccharide. The number of glycoside peaks, their relative retention time and relative proportion are characteristic of each monosaccharide, allowing to their identification. Mass spectra were also useful to confirm the final identification of each TMS methyl glycoside derivatives. For this, literature with spectral data was used (Doco *et al.*, 1999; Blenton *et al.*, 1996).

Although spectra are useful for attributing furanosides and pyranosides structures, they are of limited use for the identification of  $\alpha$ - and  $\beta$ -anomers. It is well known that the methanolic HCl medium favors the  $\alpha$ -anomer relatively to  $\beta$  one. Considering the chair conformation  ${}^4C_1$  of pentoses and hexoses, the axial position of the OH at  $C_1$  is thermodynamically more favorable and more abundant. Therefore,  $\alpha$ -pyranosides are predominant on all sugar, except for arabinose; in this case, it pyranoside form favors  ${}^1C_4$  conformation, and equatorial position is the preferred (Blenton *et al.*, 1996). These considerations were useful in the identification of each derivative.

The quantification was based on the sum of all the detected forms as summarized in the legend. Moreover, phenolic derivatives as those from gallic and ellagic acids were also identified.

#### Assessment of antitumor activity of extracts

The anti-proliferative potential of some extracts were evaluated by their effect on the viability of human breast cancer cells treated with the extracts compared to cells growing in control conditions (non-treated). Extracts were selected based on the different conditions of extraction, being the extremes conditions selected for comparison. The results are summarized in **Table 6**.

Within the studied extracts, those produced in experimental conditions unfavorable to phenolic compounds, such as low temperature and time (extract I) or using water as extraction medium (extract IX and X), demonstrated no activity against the studied carcinoma cells. On the other hand, extracts IV, VIII, XI, XII and optimum extract exhibited a significant anti-proliferative potential. Among the most effective ones, VIII

and optimal extracts revealed important activity with IC<sub>50</sub> of 92 and 176 µg/mL, respectively. Considering that extract VIII contains less TPC than optimal extract (Table 3), the increment of activity is certainly related with a very active component in the former. This subject deserves further characterization studies. The extracts IV, XI and XII presented similar potential (220-270 µg/mL).

In spite of the TPC of the extract XII being very close to that obtained at the optimal conditions, its activity is considerable lower. This result is likely related with the high temperature of extraction, which probably had led to degradation of some of the active compounds; this can also be part of the reason for the low activity registered on extract IV.

#### CONCLUSIONS

The bark of *E. globulus* has a chemical composition quite similar to the corresponding wood, nevertheless, with higher content of extractives, namely water and NaOH soluble material. Carbohydrate analysis suggests that hemicelluloses of bark are mainly composed by glucuronoxylans. The bark lignin is composed of guaiacyl:syringyl units in the proportion 80:20.

RSM was used to model and optimize the extraction of phenolic compounds from *E. globulus* bark proving to be a reliable tool in assessing the effect of three main independent variables (time, temperature and ethanol %). The optimal extraction conditions for total phenolic compounds were: 264 min, 82.5 °C, and 52% of ethanol. In the optimal extraction conditions, the experimental values found were close to the predicted ones.

The potential of *E. globulus* bark as source of valuable phenolic compounds was proven by the interesting yields achieved and by the biological activity detected. The estimated potential for the production of these compounds is about 2,356 tons/year for a mill plant with a capacity for 500 thousand tons of pulp/year. The interesting proprieties found stimulate further research studies on the separation processes to improve the extract quality and, simultaneously, a detailed chemical characterization.

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