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Extraction of bioactive compounds from the leaves of *Eucalyptus globulus* using green solvents

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Abstract

Eucalyptus globulus is a well-known source of phenolic compounds with many applications in the pharmaceutical, cosmetic, and food industries, being extensively found in Portuguese territory. Several studies have shown that the *E. globulus* extract contains important compounds for pharmacological and medicinal use with analgesic, antimicrobial, anti-inflammatory, antioxidant effects, among other applications. The use of its natural extracts has also been suggested as an alternative to synthetic drugs and additives, aiming to avoid possible undesirable side effects and prevent the development of resistance by microbiological organisms. The study of the matrix composition is essential to the development of new applications by obtaining bioactive compounds with high added value. In this work, the extraction of phenolic compounds from eucalyptus leaves was applied through heat-assisted and ultrasound-assisted techniques, using a green solvent (ethanol:water). Each extraction process was optimized using Response Surface Methodology (Traditional and Dynamic). The extraction time, solid/liquid ratio, the proportion of ethanol in the solvent, and temperature (or power, in the case of ultrasound-assisted extraction) were used as independent variables, and the extraction yield, phenolic acids, flavonoids, and total phenolic contents were the dependent variables. To support the development of new applications, bioactivity studies were carried out to evaluate the antioxidant, antimicrobial, anti-inflammatory and cytotoxicity activities of the extracts obtained under optimized conditions. Maximum antioxidant concentrations, between 5.95 to 6.85 µg/mL of dry extract, were determined by TBARS, expressed in terms of EC_{50}. The antimicrobial activity was tested against several pathogens (Gram-positive and Gram-negative bacteria), demonstrating the ability to inhibit their growth. The highest antibacterial activity was obtained, with minimum inhibition concentration values of 0.6 mg/mL, for *Yersinia enterocolitica* and 0.3 mg/mL for *Staphylococcus aureus*. For the antifungal activity, *Aspergillus fumigatus* presented the highest sensibility. The anti-inflammatory activity was confirmed by inhibition of nitric oxide release in RAW 264.7 cells (murine macrophage). The extracts did not show cytotoxicity for the cell line tested (Vero cell line). The results obtained indicate that the extracts of *E. globulus* leaves have high bioactive capacity. Further studies should be conducted to verify the expected variability in the bioactive profile over time by collecting and analyzing periodically samples from the same region and under the same conditions.

Keywords: *Eucalyptus globulus*; *extraction; green solvents; bioactivity.*

Resumo

O *Eucalyptus globulus* é uma árvore conhecida por ser uma fonte de compostos fenólicos com inúmeras aplicações na indústria farmacêutica, cosmética e alimentar, sendo extensivamente encontrada em território português. Diversos estudos têm demonstrado que o extrato de *E. globulus* contém compostos importantes para uso farmacológico e medicinal com efeitos analgésicos, antimicrobianos, antiinflamatórios, antioxidantes, entre outras aplicações. O uso de seus extratos naturais também tem sido sugerido como alternativa às drogas e aditivos sintéticos, visando evitar possíveis efeitos colaterais indesejáveis e prevenir o desenvolvimento de resistência por organismos microbiológicos. O estudo da composição da matriz é essencial para o desenvolvimento de novas aplicações a partir da obtenção de compostos bioativos de alto valor agregado. Neste trabalho, a extração de compostos fenólicos de folhas de eucalipto foi feita por meio de técnicas assistidas por calor e ultrassom, utilizando um solvente verde (etanol: água). Cada processo de extração foi otimizado usando duas estratégias de Metodologia de Superficie de Resposta (Tradicional e Dinâmica). O tempo de extração, a relação sólido/líquido, a proporção de etanol no solvente e a temperatura/potência foram usados como variáveis independentes, e o rendimento da extração, o teor de ácidos fenólicos, flavonóides e compostos fenólicos totais foram as variáveis dependentes. Os extratos foram caracterizados por análise de HPLC-DAD-ESI/MS para ácidos fenólicos e flavonóides, nos quais apresentaram alta concentração de galotaninos, flavonóides e derivados de ácidos fenólicos. Para apoiar o desenvolvimento de novas aplicações, foram realizados estudos de bioatividade a fim de avaliar as atividades antioxidante, antimicrobiana, anti-inflamatória e citotoxicidade dos extratos obtidos em condições otimizadas. Concentrações máximas de antioxidantes, entre 5.95 a 6.85 µg/mL de extrato seco, foram determinadas por TBARS, expressas em termos de EC_{50}. A atividade antimicrobiana foi testada contra vários patógenos (bactérias Gram-positivas e Gram-negativas), demonstrando capacidade de inibir seu crescimento. A maior atividade antimicrobiana foi obtida, com valores de MIC de 0.6 mg/mL, para *Yersinia enterocolitica* e 0.3 mg/mL para *Staphylococcus aureus*. Para atividade antifúngica, *Aspergillus fumigatus* apresentou maior sensibilidade. A atividade anti-inflamatória foi confirmada pela inibição da liberação de óxido nítrico em células RAW 264.7 (macrófago de camundongo). Os extratos não apresentaram citoxicidade para a linhagem celular testada (linha celular Vero). Os resultados obtidos indicam que os extratos de folhas de *E. globulus* apresentam boa capacidade bioativa. Novos estudos devem ser realizados para verificar a variabilidade esperada no perfil bioativo ao longo do tempo, por meio da coleta e análise periódica de amostras da mesma região e nas mesmas condições.

Palavras-chave: *Eucalyptus globulus*; *extração; solventes verdes; bioatividade.*

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List of acronyms

Abbreviation	Definition
ABTS	2,20-Azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid])
CaGlu	Citric Acid:Glucose
CCCD	Central composite circumscribed design
CAA	Cell Antioxidant Activity
ChE	Choline Chloride:Ethylene Glycol
ChGlu	Choline Chloride:D-(+)-Glucose
ChX	Choline Chloride:Xylitol
CIMO	Centro de Investigação de Montanha (Mountain Research Center)
DES	Deep eutectic solvents
DMEM	Gibco Dulbecco's modified eagle medium
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EC_{50}	Concentration to reduce by 50% the oxidants
EO	Essential oil
ESI-MS	Electrospray ionization mass spectrometry
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power assay
GI_{50}	Concentration with the ability to inhibit cell growth by 50%
HPLC	High-performance liquid chromatography
IC_{50}	Concentration that causes the 50% inhibition of a specified response
ICNF	Instituto da Conservação da Natureza e das Florestas (Institute for Nature Conservation and Forests)
IFN6	6° Inventário Florestal Nacional (6^{th} National Forest Inventory)
LSR	Liquid-solid ratio
MAE	Microwave assisted extraction
MBC	Minimum bactericidal concentration
MDA	malondialdehyde
ME	Maceration extraction
MEB	Malt extract broth
MFC	Minimum fungicide concentrations

MIC	Minimum inhibitory concentration
OxHLIA	Oxidative hemolysis inhibition test
PC	Phenolic compounds
RSM	Response surface methodology
SC-CO_2	Supercritical carbon dioxide
SE	Soxhlet
SFE	Supercritical fluid extraction
SLE	Solid-liquid extraction
SLE-DES	Solid-liquid extraction using deep eutectic solvents
SRB	Sulforhodamine B
TBA	2-Thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Substances assay
TEAC	Trolox equivalent antioxidant capacity parameter
TFC	Total flavonoid content
TPA	Total phenolic acid content
TPC	Total phenolic content
TSB	Tryptic Soy Broth
UAE	Ultrasound-assisted extraction

1. Scope and objectives

1.1. Eucalyptus: geographical, economical, and biological aspects

Eucalyptus, a member of the Myrtaceae family, is one of the most exploited trees in the world, with an occupied global area estimated at 16 to 19 million hectares (Rodrigues *et al.*, 2018a). Although this tree is widely grown in many countries all over the world, most of the species are native to Australia, being extensively cultivated in subtropical and Mediterranean regions as Portugal and Spain (Fernández-Agulló *et al.*, 2015; Liu *et al.*, 2016; Rodrigues *et al.*, 2018b).

Figure 1.1: *Eucalyptus globulus* tree and leaf from Águeda, Portugal.

Forest surface data of continental Portugal presented in the 6th National Forest Inventory (IFN6) from Nature Conservation Institute and Forestry (ICNF), show that, in the Northern region of the country, there were 145.4 kha accounted in 2015, being, therefore, the third region with the largest eucalyptus forest population, behind the Central region (402 kha) and Alentejo (181.6 kha) (Instituto Nacional de Estatística, 2019). In the same year, the most represented tree in continental Portugal was Eucalyptus, with 25.7%, followed by Cork Oak with 23.6% and Pine Tree with 20.6% (ICNF, 2019).

Given its large availability in Portuguese territory, eucalyptus is also widely exploited by the industry, being the main raw material of the Portuguese pulp industry (Branco & Neves, 2018). It is extremely important to find new applications and uses for the eucalyptus trees remains, as, for example, the extraction of high added value compounds from their leaves.

In 2016, the Grand View Research, a U.S. based market research and consulting company, foreshadowed an increasing demand in the global polyphenol market, affirming that in 2018 this sector was valued at USD 1.28 billion and was expected to register an estimated compound annual growth rate of 7.2% from 2019 to 2025 (Grand View Research, 2019). In this context, the extraction of bioactive compounds, as phenolic compounds (PC), from eucalyptus leaves may be an interesting path of valorization of this biomass (Vecchio *et al.*, 2016).

Recent studies have demonstrated antimicrobial, antifungal and antioxidant properties for extracts of wood, bark and leaves, and essential oils (EOs) of *Eucalyptus* species (Fernández-Agulló *et al.*, 2015; González-Burgos *et al.*, 2018; Vázquez *et al.*, 2008). Extracted compounds from this plant have also been used as flavor and aroma enhancers in food and as active ingredients for sanitary products and cosmetic formulations (Gilles *et al.*, 2010; V. de S. Mota *et al.*, 2015). The in-depth study of the matrix composition of *Eucalyptus* species is a fundamental step for its maximum use in industrial applications and to obtain bioactive compounds with high added value. In addition, this strategy is coherent with the emerging concept of biorefinery, a term of increasing interest, intending to promote the usage of biomass resources in the search for eco-friendly alternatives to some traditional products and processes, as well as a zero-waste economy (Neiva *et al.*, 2018).

Given its importance in the field of health, technology, and sustainability, and the large plantation available in the country, there is a great interest in studying and exploring these species, isolating, and purifying their high-value bioactive compounds, and contributing efficiently and sustainably to add value to this biomass.

1.2. Objectives

The main objective of this work is the optimization of the extraction process of phenolic compounds from the leaves of *Eucalyptus globulus* using green solvents and evaluating the bioactivity of the extracts obtained under optimized conditions, aiming to valorize this biomass, and contributing to the development of potential applications in the food, pharmaceutical, and cosmetics areas.

To accomplish this, the following tasks are foreseen:

- Establish experimental design methodologies that will be applied to optimize the extraction process, using two techniques: heat-assisted extraction and ultrasound-assisted extraction, and considering the application of water, ethanol, and eutectic systems as solvents;
- Carry out the extraction process in the laboratory, identifying and quantifying the phenolic composition of the extracts, by high-performance liquid chromatography (HPLC);
- Model and evaluate the effects of the studied variables on the bioactive compounds recovery and extraction yield using the Response Surface Methodology (RSM);
- Explore different approaches of Response Surface Methodology combined with optimization and statistics procedures;
- Determine the cytotoxic, antimicrobial, antioxidant, and anti-inflammatory activities of the extracts obtained under the optimized conditions.

2. State of Art

2.1. *Eucalyptus globulus*

The *Eucalyptus* genus comprises about 900 native species and subspecies, mainly from Australia. One of its most well-known species is *Eucalyptus globulus*, native to southeastern Australia and Tasmania, but also widely cultivated in the Mediterranean region. Since the mid-19th century, it has been successfully introduced in 90 countries around the world (Vecchio *et al.*, 2016), including Portugal and Spain (Fernández-Agulló *et al.*, 2015).

E. globulus is, in fact, the most planted eucalyptus species in Portugal due to its comparative advantage in terms of adaptability to the climate and terrain, high growth rate, and quality of its wood. According to historical records, the first seeds of *E. globulus* and other eucalyptus species would have arrived in Portugal in the early 1850s. A few years later, the use of eucalyptus as an ornamental plant was already frequent, especially in Lisbon gardening and landscaping and in the southern region of the country. However, the expansion of the eucalyptus forested area occurred mainly between the 60s and 90s (Águas *et al.*, 2017; Alves *et al.*, 2007; Cabral, 2019). The eucalyptus production has been increasing since then, given its applicability in the industry, being the main raw material in the production of cellulose and paper and, consequently, its residues constitutes most of the waste generated (Santos *et al.*, 2011).

Besides its application in the paper industry, eucalyptus trees play a very important role as a source of bioactive molecules, among them terpenoids, tannins, flavonoids, and phloroglucinol derivatives (Boulekbache-Makhlouf *et al.*, 2013). Teixeira *et al.* (2019), reported the characterization of the phenolic content from aqueous leaf extracts, revealing a high concentration of gallotannins, phenolic acids, and flavonoids.

Because of the high amount of bioactive compounds, hydroethanolic *E. globulus* leaf extracts have been defined as having antiseptic properties and used to treat respiratory tract infections, flu, and other respiratory complications (Dezsi *et al.*, 2015; World Health Organization, 2002). Dezsi *et al.* (2015) attested positive responses to the use of eucalyptus extract to treat infections caused by both Gram-negative bacteria (*Salmonella typhimurium* and *Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis,* and *Listeria monocytogenes*). Teixeira *et al.* (2019), on the other hand, demonstrated the potential anti-cancer cell growth inhibitory effect of *Eucalyptus globulus* aqueous extracts,

being able to hinder the cell growth of three different human tumor cell lines (NCI-H460, PANC-1, and HCT-15[1]). *E. globulus* leaves extracts have also been used as food additives (Boukhatem *et al.*, 2020), adopted in perfumery, cosmetics, aromatherapy, and phytotherapy (Vecchio, Loganes, and Minto 2016; Ait-Ouazzou *et al.* 2011).

Currently, there is pressure from the market to reduce and/or replace synthetic products that can cause undesirable side effects, coupled with new proposals for bioeconomics and sustainability trends. Natural unaltered products and their derivatives head a substantial market share of pharmaceutical products, comprising 48% of antibacterial agents, 12% of antifungal and antiviral agents, 35% of antiparasitic agents, and 53% of anticancer compounds, approved by legislation in the last 40 years (Newman & Cragg, 2020). The substances present in these products, mostly from medicinal plants, can be considered bioactive and potentially developed as pharmacological drugs. Therefore, researchers and manufacturers have been looking for alternatives in products of natural origin, mainly from plant extracts, with proven benefits (Ait-Ouazzou *et al.*, 2011; Carocho *et al.*, 2015; Dezsi *et al.*, 2015).

2.2. Extraction techniques

According to Pena-Pereira and Tobiszewski (2017), different extraction techniques allow the isolation and purification of target compounds, phase transfer of target molecules, and sample cleanup. Although conventional extraction techniques are widely described in the literature for the extraction of bioactive compounds from *E. globulus* leaves, bark, flower, and wood (Table 2.1), there is still little information regarding sustainable extraction methodologies or apply green solvents in their studies.

In general, the techniques reported are processes suited for laboratory scale, thus evidencing the need to deeply study a sustainable process that can be extended to industrial level. Furthermore, comparisons of extraction methods usually discuss only relative recoveries of target analytes, some physical conditions as pressure and temperature, and the amount of organic solvent required, and tend to ignore other important chemical characteristics of the extraction method. The selectivity of an extraction method for target analytes in a plant matrix is important to consider, as there may be co-extracted chemical

[1] Colorectal, pancreatic and non-small cell lung cancer, respectively.

compounds, often requiring post-extraction purification steps prior to chromatographic analysis (Hawthorne *et al.*, 2000).

Some of these traditional techniques have been used for more than a century – *e.g.* solid-liquid extraction (SLE), Soxhlet (SE), percolation, and extraction by maceration (ME) (Ameer *et al.*, 2017). However, these techniques have some disadvantages due to the excessive consumption of time, energy and, sometimes, due to the use of polluting solvents (Bubalo *et al.*, 2018).

The infeasibility of certain traditional methods to be applied at an industrial scale has triggered researchers to find more efficient and ecological techniques for extracting active compounds from a wide range of plant matrices and their components (Ameer *et al.*, 2017), so-called green extractions. A few alternatives to conventional techniques have been proposed for the extraction of target compounds from various matrices, including ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE). These techniques offer excellent potential to reduce or eliminate the use of toxic chemical solvents while improving process efficiency and enhancing both extraction yields and the quality of the extract (de la Guardia & Armenta, 2011; Pena-Pereira & Tobiszewski, 2017).

Table 2.1: Bibliographic summary of the techniques and conditions applied to the extraction of bioactive compounds from *Eucalyptus* leaves.

Technique	Extraction condition					Total extraction yield	References
	Solvent and proportions		Temperature	Time	other		
	-	(%)	(°C)	(min)	-	η_{total} (%)	
Supercritical Fluid Extraction (SFE)	EtOH:H_2O (with CO_2)[i]	5:95	40	360	250 bar	3.95	(Rodrigues *et al.*, 2018b)
	CO_2: EtOH	95:5	40	360	200 bar	3.16	(Rodrigues *et al.*, 2018a)
	CO_2: EtOH	95-85:5-15	80	120	50 MPa	5.97	(Zhao & Zhang, 2014)
Microwave-assisted Extraction (MAE)	$[HO_3S(CH_2)_4mim]HSO_4$: H_2O	1:56[v]	-	20	385 W	4.82	(Liu *et al.*, 2016)
	EtOH:H_2O	56:44	-	7	Low[ii]	ns	(Gullón *et al.*, 2019)
	H_2O	-	-	3	600 W	ns	(Bhuyan *et al.*, 2015)
Sohxlet	DCM	ACS	-	360	-	7.32	(Rodrigues *et al.*, 2018a)
	EtOH	ACS	-	360	-	25.90	(Rodrigues *et al.*, 2018a)
	MeOH	ACS	-	360	-	30.34	(Rodrigues *et al.*, 2018a)
	EtOH	ACS	90	480	-	36.33	(Zhao & Zhang, 2014)
	Hexane	ACS	78	480	-	9.3	(Zhao & Zhang, 2014)
Maceration (ME)	C_6H_{14}: C_3H_6O	50:50	50	3	-	5.66	(Rodrigues *et al.*, 2018a)
	n-Hexane: C_3H_6O	50:50	50	3	-	5.16	(Rodrigues *et al.*, 2018a)
	EtOH:H_2O	80:20	50	300		35.7	(Gullón *et al.*, 2017)
	EtOH:H_2O	56:44	50	225	-	32.7	(Gullón *et al.*, 2019)
	EtOH:H_2O	70:30	-	180	-	16.36	(González-Burgos *et al.*, 2018)
	MeOH:H_2O	70:30	-	180	-	39.57	(González-Burgos *et al.*, 2018)
	C_3H_6O:H_2O	70:30	-	180	-	35.86	(González-Burgos *et al.*, 2018)

Table 2.1: (continued).

	DCM	ACS	-	1440	-	33.1[iii]	(Mworia *et al.*, 2019)
	EtOH:H_2O	52:48	82.5	264	-	ns	(I. Mota *et al.*, 2012)
	C_3H_6O: H_2O	70:30	-	-	10080[iv]	ns	(Boulekbache-Makhlouf *et al.*, 2013)
Solid-Liquid Extraction using Deep Eutectic Solvents (SLE-DES)	ChE: H_2O	82:20	50	60	-	ns	(Gullón *et al.*, 2019)
	ChX	5:1[v]	50	60	-	ns	(Gullón *et al.*, 2019)
	ChGlu	1:1[v]	50	60	-	ns	(Gullón *et al.*, 2019)
	CaGlu	1:1[v]	50	60	-	ns	(Gullón *et al.*, 2019)
Hidrodestilation (HDT)	H_2O	-	-	300	-	3.77	(Zhao & Zhang, 2014)
Enzime-assisted Extraction (EAE)	EtOH:H_2O Comercial Cellulase	56:44 5:95[vi]	50	225 30	-	ns	(Gullón *et al.*, 2019)
Ultrasound-assisted Extraction (UAE)	H_2O	-	60	90	250 W	ns	(Bhuyan *et al.*, 2017)
	EtOH:H_2O	56:44	50	90	40 kHz	ns	(Gullón *et al.*, 2019)

Petroleum Ether (C6H14O), Acetone (C3H6O), Ethanol (EtOH), Water (H2O), Dichloromethane (DCM), Methanol (MeOH), Brönsted acidic ionic liquid ([HO3S(CH2)4mim]HSO4), Carbon dioxide (CO2).
ns - not specified by the author(s).
[i] Assisted by 12gCO2.min-1.
[ii] The author did not specify the power value, citing as “low, medium-low and medium” in the article.
[iii] This study only considered the extraction of antipyretic compounds.
[iv] The process of extraction was performed for a week at room temperature in the dark, using a magnetic stirrer.
[v] Molar ratio.
[vi] Enzyme-solid ratio (ESR, v:w).

2.2.1. Conventional extractions

Conventional extractions are widely used by authors to compare results from pioneering and innovative techniques in obtaining compounds from plant matrices, although traditional techniques, such as some Maceration and Soxhlet, can be usually time-consuming processes, demanding larger volumes of solvent and are not consistent with sustainable proposals, considering that organic solvents are often used in these processes.

Maceration extraction (ME)

Maceration (ME), a type of solid-liquid extraction, occurs when a solute is extracted from a solid phase with the help of a solvent. The process overall involves the penetration of a certain amount of solvent into the solid in question, the dissolution of the soluble components, the transport of solutes from the interior of the solid particles to their exterior by diffusion and dispersion of the solutes within the solvent phase. In some cases, a solubilization step may include transformed changes promoted by the solvent, such as hydrolysis of insoluble biopolymers to produce soluble molecules (Berk, 2009).

Several studies concerning the extraction of bioactive elements from plants and herbs have been published considering the effects of time, temperature, and type of solvent for ME processes (Table 2.1). Mota *et al.* (2012), for example, studied the influence of time (30−360 min), temperature (25−140 °C), and ethanol concentration (0−80%) in solid-liquid extraction of compounds from *E. globulus* bark, obtaining a yield of 21.7 wt% with optimal conditions. In another study, Gullón *et al.* (2019) considered also the effect of particle sizes in obtaining phenolic compounds, varying particles between 0.5 to 2 mm. Particles of grounded *E. globulus* leaves were submitted to 25 to 50 °C temperature, 120 rpm stirring frequency, using aqueous ethanol as solvent (20 to 80%, v:v) during 30-300 min, based on the previous study of the same authors (Gullón *et al.*, 2017).

Soxhlet extraction (SE)

Among the techniques regarding conventional solid-liquid extractions, Soxhlet has been the most widely used leaching technique for a long time and the main reference in performance analysis for other leaching methods (Castro & García-Ayuso, 1998).

The structure of the Soxhlet has three parts: a glass reservoir, also known as a *thimble*, which contains a tube on the side where the material is loaded or unloaded; the condenser, responsible for the reflux of the solvent; and the flask, set up at the bottom and distills the compounds with the addition of solvent. In a conventional Soxhlet, the sample is placed in a thimble extractor and, during the operation, gradually filled with fresh solvent condensed from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the thimble solute and discharges it back into the distillation flask, taking the extracted analytes into the liquid. This operation is repeated until complete extraction is achieved.

The design of new rapid extraction techniques, for example, MAE and SFE, relegated the conventional Soxhlet as an old-fashioned, time-consuming, and manual technique (Castro & García-Ayuso, 1998). Although updated by the incorporation of auxiliary energies, such as the introduction of microwaves, SE is still time-consuming and requires large amounts of solvent (Castro & García-Ayuso, 1998; Hawthorne *et al.*, 2000).

Rodrigues *et al.* (2018a) performed preliminary wax removal procedures for *E. globulus* leaves to later proceed to the Soxhlet. For dewaxing, the cut leaves (60 g) were immersed during a short period of 30 seconds in a glass flask containing a mixture of organic solvents (600 mL) at 50 °C. The yields obtained using dewaxing treatment were higher compared to extractions without pre-treatment. Therefore, it was confirmed that *E. globulus* leaves contain significant amounts of cuticular wax that can hinder mass transfer during extraction. In addition, the experiment showed significant variability, with total yields for extractions with polar solvents (25.90 wt% ethanol and 30.34 wt% with methanol) in contrast to dichloromethane (7.32 wt%). The authors also evaluated the concentration of triterpenic acids in the extract, obtaining 5.39, 7.79, and 1.34 (wt%) using ethanol, methanol, and dichloromethane as solvent, respectively.

Zhao and Zhang (2014) reproduced the extraction in Soxhlet using 5 g of chopped eucalyptus leaves immersed in ethanol or hexane at 90 °C for a variable time up to 8 h. The authors used response surface methodology to achieve optimal extraction parameters, and it was found that the solvent has a more significant effect compared to the extraction time. In this case, the highest total yield was found for ethanol at about 36.33 wt%, containing terpenes as major compounds. Some Soxhlet extractions of *E. globulus* leaves are summarized in Table 2.1.

2.2.2. Green extractions

Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction (UAE) is based on the principle of particle vibration employing mechanical oscillating sound waves with an incidence in an elastic medium, with a frequency between 20 kHz and 10 MHz above the limit for human hearing detection. The main physical parameters of ultrasound include power, frequency, and amplitude (Vernès *et al.*, 2019).

The introduction of ultrasonic waves of certain frequencies (20-1000 kHz) can lead to sudden pressure changes in alternating cycles of compression and rarefaction together with the creation of bubbles followed by events of adiabatic collapse and shock waves. In the UAE case, these collapses are called acoustic cavitations, considering that a passage of ultrasound waves occurs in the liquid medium (Panda & Manickam, 2019). This phenomenon can generate temperatures up to 5000 K inside the *nucleus* of the bubbles, together with a production of highly reactive free radicals due to the homolytic cleavage of water molecules and dissolved gases, such as oxygen. The formation of transient bubbles and successive collisions generate some physical effects in the environment, such as turbulence, shear conditions, shock waves, and microjets (Esclapez *et al.*, 2011; Panda & Manickam, 2019).

Depending on the intensity level, different effects can be achieved. At low intensities, such as external and possibly internal mass transfer resistances are affected. However, a product structure remains largely unchanged. Intermediate intensities can affect a product structure, thereby increasing the effects on resistance to internal mass transfer. If the ultrasonic power still increases further, the cell may disintegrate (Esclapez *et al.*, 2011).

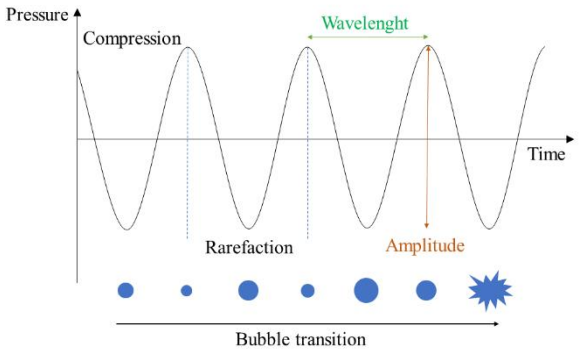


Figure 2.1: Ultrasonic parameters related to cavitation phenomenon (adapted from Vernès, Vian, and Chemat, 2019).

According to Panda and Manickam (2019), the extraction of natural products through cavitational effects demonstrated increasing mass transfer rate and greater penetration of solvent into the cell walls of the plant matrix, due to the temperature and pressure during the collapse of the bubbles. In addition, an intense interparticle collision and increased contact surface area through the disintegration of the matrix by shock waves and solvent microjets should occur. Therefore, the use of techniques with potential mass transfer, better solvent penetration, and less consumption of toxic solvents, as mentioned above, may be carried out with lower price range, faster extraction rates, and greater yield content in the extraction of bioactive compounds in plant matrices, as reported by several authors (Aspé & Fernández, 2011; Caleja *et al.*, 2017; Gullón *et al.*, 2019; Pinela *et al.*, 2019; Vinatoru, 2001).

Gullón *et al.* (2019) and Bhuyan *et al.* (2015) assessed conditions for the ultrasound-assisted extraction for *Eucalyptus* leaves testing different ranges of temperature, frequency, and type of solvent. According to Bhuyan *et al.* (2015), the temperature had the greatest impact on the total phenolic content and yield followed by time and power. A yield of approximately 163 mg of gallic acid equivalents/g of total phenolic content was observed using 250 W for 90 min at 60 °C using water as solvent. However, in Gullón *et al.* (2019), maintenance of ultrasonic cavitation for a longer period resulted in a reduction in all performance indicators evaluated. In this case, the phenolic content of the extracts obtained after 120 min of UAE was 15% lower than that of the 90 min extraction, and the antioxidant activity was even lower, showing a 37% reduction. Gullón *et al.* (2019) reported the following optimal conditions: 56% ethanol (v:v), LSR of 10 mL/g, and 50 °C. The extraction was carried out in an ultrasonic bath, with temperature and time control, working at a

frequency of 40 kHz, fitting six flasks at the same time. Different extraction times from 15 to 120 min were evaluated and the optimal time was 90 min of extraction (Table 2.1).

Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) involves the use of energy from microwaves – non-ionizing electromagnetic waves that have a frequency of 300 MHz to 300 GHz – to heat a solvent in contact with a sample to transfer target compounds from the sample matrix to the solvent. MAE is based on system heating due to the absorption of microwave energy by polar molecules and has been successfully applied in several areas since the published research by (Ganzler *et al.*, 1986). MAE efficiency depends on factors such as solvent, sample material, target components, and the respective dielectric constants. The greater the dielectric constant, the greater the amount of energy absorbed by the molecules, therefore, the faster the system reaches the optimal extraction temperature (de la Guardia & Armenta, 2011).

Microwave heating is based on two principles: ion conduction and dipole rotation. Ionic conduction refers to the electrophoretic migration induced by charge carriers, such as ions and electrons, by the influence of the electric field produced via incident microwaves. Dipoles are molecules with polarized bonds due to the difference in electronegativity between atoms. When there is no electric field, the molecules are randomly oriented. When an alternating electric field is applied to the medium during MAE, the dipoles are oriented according to the direction of the field (induced dipole moment) and disorientated when the field is canceled (Figure 2.2) (Vernès *et al.*, 2019). The dipole's rotation happens multiple times since the dipoles always try to align with the imposed alternating electric current. The oscillation of these dipolar species leads to collisions between particles and, therefore, generates heat. Because of the heat generated from inside the system, the pressure inside the plant matrix increases and the cell membrane disrupts, allowing an easier solvent penetration (Vernès *et al.*, 2019).

The MAE is suitable for rapid analysis and offers a considerable reduction in time and amount of solvent. In addition, regarding the extraction of organic compounds, the MAE offers a high sample yield compared to the Soxhlet, except for thermally unstable compounds due to the fast increase of internal temperature (de la Guardia & Armenta, 2011; Pena-Pereira & Tobiszewski, 2017).

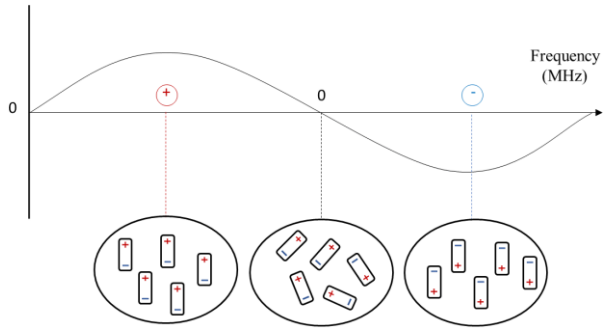


Figure 2.2: Dipole orientation in the cell according to microwave alternating electric field (adapted from Pena-Pereira and Tobiszewski 2017).

Gullón *et al.* (2019) evaluated the extraction of phenolic compounds and antioxidant activity from powdered leaves of Eucalyptus species using microwaves. The extraction occurred using aqueous ethanol as a solvent (56%, v:v) and LSR of 10 mL/g. Different power levels (low, medium-low and medium, according to the authors) and irradiation periods (2 – 7 min) were evaluated (Gullón *et al.*, 2019). Bhuyan *et al.* (2015) and Liu *et al.* (2016) also studied MAE of eucalyptus leaves and obtained the optimal conditions: 3 min, 600 W, LSR 50 mL/g and water as solvent; and 20 min, 385 W, LSR 50 mL/g, and Brönsted acidic ionic liquid (1.0 mol/L), respectively. Bhuyan *et al.* (2015) obtained a total phenolic content of 58.40 mg of galic acid equivalents/g dw, and a total flavonoid content of 19.1 mg rutin equivalents/g dw, lower values than those obtained by Gullón *et al.* (2019). These authors took into consideration the influence of the three independent parameters: irradiation time, power, and type of solvent (Bhuyan *et al.*, 2015; Gullón *et al.*, 2019; Q. Wang *et al.*, 2016). More details are provided in Table 2.1.

Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) involves using a solvent at a temperature and pressure above its critical values. The qualitative and quantitative composition of the final extract is provided by the physicochemical properties of the solvent and by the process parameters, such as temperature, pressure, and extrinsic aspects, such as the characteristics of the sample matrix and environmental factors. Small changes in the extraction parameters can result in major alterations in the extraction properties and the efficiency of the extraction (Wrona *et al.*, 2017). Therefore, the proper control of the parameters in SFE is crucial for obtaining a high value-added product. Carbon dioxide (CO_2) is the most used solvent in SFE.

Supercritical CO_2 (SC-CO_2) is a hydrophobic and non-polar solvent, so it can dissolve small molecules with low polarity. By adding portions of a polar co-solvent, the properties of the mixture can be manipulated to include polar compounds in the extract (Q. Wang *et al.*, 2016).

A pressure-temperature phase diagram, shown in Figure 2.3, illustrates the nature of a supercritical fluid. In SFE, the process parameters must be higher than the parameters at the critical point. These values, namely critical temperature and critical pressure are intrinsic properties of any substance. When the solvent is in the supercritical region, it exhibits intermediate properties of gas and liquid, so it is possible to change the properties of the solvent, for example its diffusivity viscosity or surface tension, by minimally changing the process parameters. Thus, optimal extraction conditions are achieved due to the ease and speed of mass transfer (Q. Wang *et al.*, 2016).

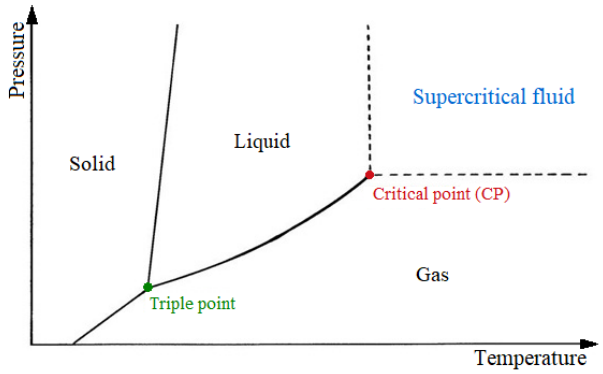


Figure 2.3: Generic pressure-temperature (P-T) phase diagram for a pure compound.

Therefore, the productivity and profitability of an SFE process depend a lot on the selection of the process parameters. Solubility, which must be high in supercritical extraction, is the most important criterion that affects the efficiency of extraction and the qualitative composition of the final product. The objective of the extraction of plant material is to obtain the largest possible amount of extract rich in desirable active compounds in a fast, viable, and cost-effective way.

Several studies evaluated the efficiency of obtaining bioactive compounds from eucalyptus species. In Rodrigues *et al.* (2018b and 2018a), extracts of *Eucalyptus globulus* leaves were obtained by extraction with supercritical carbon dioxide. For the SFE tests, the best results were obtained with CO_2 modified with 5.0% by weight of ethanol, at 200 bar

and 40 °C, in which the yield obtained was 3.95 wt%. According to the authors, the recovery of bioactive compounds in this study are essentially independent of pressure, however, the ethanol content in the solvent has a very positive impact on the process.

2.3. Chemical profile of *Eucalyptus globulus* extracts

Studies addressing phytochemical screening of extracts and essential oil of *E. globulus* showed the presence of several classes of compounds such as flavonoids, terpenoids, saponins, tannins, phenolic acids and reducing sugars. The presence of many secondary metabolites may be responsible for the bioactivity of this plant.

The composition of plant extracts may vary according to geographic and climatic conditions, for example (Caleja *et al.*, 2017; Mahumane *et al.*, 2016). Seasonal variations in the proportions of major and minor compounds may occur associated with physiological processes, growth processes, type age of the leaves and other environmental conditions (He *et al.*, 2000). In addition to these factors, primary treatments, methods, and conditions of extraction and even the way of drying and storing the extract can alter the composition. In-depth studies are significant to determine the best methods and conditions to obtain extracts rich in target compounds and of high added value. Given the complex chemical composition of *E. globulus*, this section will be focused especially on the approach of major classes of phenolic compounds present in eucalyptus leaves: phenolic acids, flavonoids, and tannins.

Phenolic compounds in Eucalyptus globulus

Phenolic compounds (PC) are an important group of small molecules present in plant extracts and the most abundant secondary metabolites of plants (Škrovánková *et al.*, 2012), playing a prominent role in general strategies of plant defense. PC can be divided into different subgroups based on their chemical structure as phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids (Agati *et al.*, 2012). Phenolics have one or more aromatic rings and one or more hydroxyl groups that are likely to donate a hydrogen atom or an electron to a free radical; therefore, they have an ideal structure for free radical scavenging activities.

Phenolic acids can be divided into two groups: hydroxybenzoic acids (gallic, vanillic and protocatechuic acids) and hydroxycinnamic acids (coumaric, caffeic, and ferulic acids) (Figure 2.4) with caffeic acid being the most abundant phenolic acid (Vincente *et al.*, 2009).

Flavonoids occur in medicinal plants mainly as flavones and flavonols (Figure 2.4), with the flavan core as the basic structure. Flavonoids are divided into six subgroups: flavones, flavonols, flavones, flavanones, isoflavones, and anthocyanins, according to the degree of oxidation (oxidation state) of the oxygen heterocycle (Hernández-Rodríguez *et al.*, 2018).

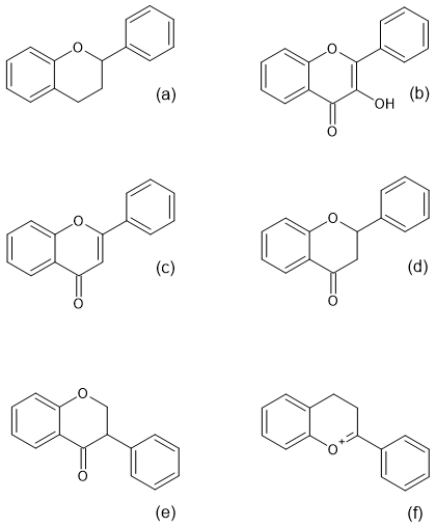


Figure 2.4: Flava core (a) and basic structures of flavonols (b), flavones (c), flavanones (d), isoflavones (e), and anthocyanidins (f).

Tannins are generally defined as complex phenolic substances of plant origin, chemically defined as phenylpropanoid compounds which are often condensed into polymers of variable length. Tannins are divided into two groups: hydrolysable and condensed (flavan-3,4-diol). Hydrolysable tannins are also subdivided into two categories: gallotannins (derived from gallic acid) and ellagitannins (derived from ellagic acid).

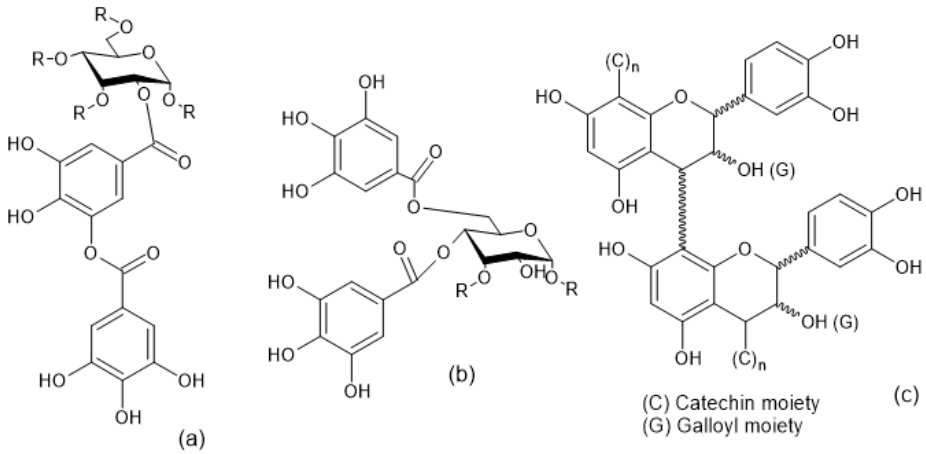


Figure 2.5: Basic structure of gallotannins (a), ellagitannins (b) and condensed tannins (c).

According to (Boulekbache-Makhlouf *et al.*, 2013), the chemical profile of *E. globulus* leaves presents a wide range of phenolic compounds, in greater quantity phenolic acids (gallic acid and derivatives), flavonoids (quercetin derivatives) and hydrolysable tannins. Similar profiles were obtained from different authors (Batish *et al.*, 2008; Gilles *et al.*, 2010; Mworia *et al.*, 2019).

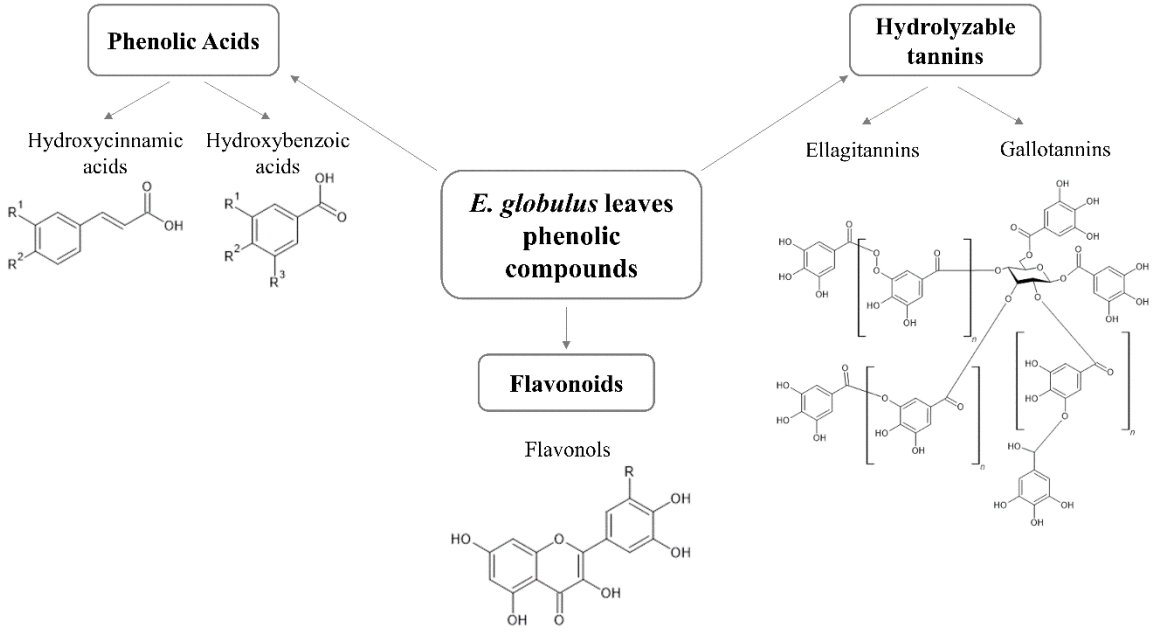


Figure 2.6: Classification of phenolic compounds from *E. globulus* leaves extract from Boulekbache-Makhlouf *et al.* (2013).

Given the importance of phenolic compounds in health and food science, some authors have reported methods of screening, recovery, and quantification of total phenolic content (TPC) for eucalyptus extracts using different extraction techniques, as shown in Table 2.2.

Table 2.2: Total phenolic content (TPC) in *E. globulus* leaves extract through heat-assisted and ultrasound-assisted extraction under different conditions.

Extraction	**Solvent** (%)	**Temperature** (°C)	**Time** (min)	**TPC** (mg/g dried leaf)	**Reference**
ME	Ethanol (56)	50	225	92.9	(Gullón *et al.*, 2017)
ME	Ethanol (56)	50	225	87.9	(Gullón *et al.*, 2019)
ME	Acetone (70)	25	180	35.9	(González-Burgos *et al.*, 2018)
ME	Ethanol (70)	25	180	16.4	(González-Burgos *et al.*, 2018)
ME	Methanol (70)	25	180	39.6	(González-Burgos *et al.*, 2018)

Table 2.3: (continued).

ME	Water (100)	50	-	113.7	(Dudonné *et al.*, 2009)
ME	Water (100)	100	5	157.4	(Teixeira *et al.*, 2019)
ME	Methanol (80)	25	60	173	(Gomes *et al.*, 2018)
ME	Propanone (70)	25	10080[i]	131.59	(Boulekbache-Makhlouf *et al.*, 2013)
ME	Ethanol (50)	25	1440	40.1	(Bajpai *et al.*, 2005)
Extraction	**Solvent** (%)	**Power**	**Time** (min)	**TPC** (mg/g dried leaf)	**Reference**
UAE	Ethanol (56)	Low[ii]	90	84	(Gullón *et al.*, 2019)

[i]Equivalent to one week.
[ii]Power was not numerically specified by the authors.

In Teixeira *et al.* (2019), infusions were prepared using 1 g of the plant material with 200 mL of distilled boiling water (100 °C), allowed to infuse for 5 min at room temperature. Eighteen polyphenols were detected, seven of which were flavonoids (quercetin, isorhamnetin, and myricetin derivatives), three phenolic acids (chlorogenic acid and ellagic acid derivatives), and eight gallotannins derivatives.

Gomes *et al.*(2018) macerated 1 g of leaves in 30 mL of methanol:water (80:20, v/v) at 25 °C under agitation (150 rpm) for one hour. The extract was redissolved at a concentration of 5 mg/mL with 80% methanol. The authors identified nineteen phenolic compounds, sixteen of which were flavonoids (mainly quercetin derivatives) and three phenolic acids (mainly gallic and ellagic acids derivatives). Digalloyl-glucoside, 5-*O*-caffeoylquinic acid, and ellagic acid glucoside were the most abundant molecules present in *Eucalyptus globulus* methanol:water extract.

Both studies determined the phenolic profile by HPLC (280, 330, and 370 nm), and the chromatographic characteristics of the main tentatively identified phenolic compounds in *E. globulus* extracts are summarized in (Table 2.4).

Table 2.4: Tentative identification and quantification (mean value ± standard deviation) of the phenolic compounds present in the extracts of *Eucalyptus globulus*.

Tentative identification	**Quantification (mg·g^{-1} extract)**	
	Teixeira *et al.* (2019)	Gomes *et al.* (2018)
Digalloyl-glucoside	34.6 ± 0.9	44.2 ± 1.3
5-*O*-Caffeoylquinic acid	18.5 ± 0.8	22.3 ± 0.3
Trigalloyl-glucoside	18 ± 0.4	23.9 ± 1.4
Myricetin-*O*-glucuronide	4.62 ± 0.08	1.90 ± 0.03
Ellagic acid glucoside	19.0 ± 0.4	21.6 ± 0.3
Tetragalloyl-glucose	5.4 ± 0.2	17.4 ± 0.8
Quercetin-3-*O*-glucuronide	4.1 ± 0.1	7.7 ± 0.2
Quercetin-3-*O*-glucoside	4.48 ± 0.05	3.7 ± 0.1
Eucaglobulin/Globulusin	19.2 ± 0.8	13.9 ± 0.4
Quercetin-*O*-pentoside	1.33 ± 0.01	1.34 ± 0.01
Quercetin-*O*-rhamnoside	2.80 ± 0.03	3.2 ± 0.1
Isorhametin-*O*-rhamnoside	1.82 ± 0.04	1.16 ± 0.02
Methylellagic acid pentoside	3.0 ± 0.1	3.2 ± 0.1
Quercetin derivative	1.16 ± 0.02	1.47 ± 0.02
Total phenolic content	**138 ± 3**	**173 ± 4**

Other authors evaluated different parts of *E. globulus* and obtained some similar compounds in their extracts. Santos *et al.* (2011), for example, described the phenolic content in methanolic, water, and methanol-water bark extracts of *E. globulus* by HPLC-UV and HPLC-MS/MS. The authors identified some phenolic acids (gallic, protocatechuic, chlorogenic, and caffeic acids), flavonoids glycosides (quercetin and isorhamnetin derivatives), and ellagic acid derivatives. Fernández-Agulló *et al.* (2015) evaluated aqueous ethanolic wood extract at 50 °C and analyzed the PC content by HPLC, obtaining a profile rich in galloylglucoses, gallic acid, ellagic acid, and quercetin-3-*O*-rhamnoside by tentative identification.

2.4. Bioactivity in extracts from *Eucalyptus globulus*

Medicinal plants can produce active ingredients that help and stabilize the functioning of organs and systems. Their extracts can serve as precursors of semi-synthetic and homeostatic drugs in the case of diseases, as well as in the control of microorganisms (Furtado *et al.*, 2015). It is estimated that 10 to 53 thousand plants are used for medicinal purposes, however, only a small part is investigated indeed, representing 1% of the flora, according to Mazzari and Prieto (2014). Currently, there is an incentive on the scientific community for experimental research to identify and quantify the bioactive potential of these plants, with a direct relationship with the antimicrobial resistance to existing synthetic drugs and the implementation of new agents against infections. In this context, bioactive compounds can also act synergistically with other substances and enhance antibiotic and antioxidant treatments.

Several studies on the bioactivity of eucalyptus residue (leaves, bark, and wood) have been published in recent years. Significant antioxidant and antimicrobial effects have also been observed for extracts from eucalyptus species, demonstrating the importance of this field of research.

2.4.1. Antibacterial activity

According to Ostrosky *et al.* (2008), different methods can be used to evaluate the antibacterial activity of the extracts, the following being the most well-known: agar diffusion method, disk-diffusion, and microdilution methods, which are carried out in broth medium. In the determination of the Minimum Inhibitory Concentration (MIC) or Minimum Bactericidal Concentration (MBC) of plant extracts, the broth microdilution method has been used more frequently. MIC is defined as the lowest extract concentration capable of inhibiting microbial growth after 24 h incubation at 37 °C, and MBC values are the lowest extract concentration that results in bacterial death after 24 h incubation at 37 °C. In these tests, reductions in bacterial growth in samples with extract compared to positive controls are assessed by comparing viable cell counts after a given incubation period (Ostrosky *et al.*, 2008).

E. globulus extracts were evaluated against several Gram-positive and Gram-negative bacterial strains, and fungi species by different authors (Ait-Ouazzou *et al.*, 2011; Batish *et al.*, 2008; Dezsi *et al.*, 2015; Fernández-Agulló *et al.*, 2015; Gilles *et al.*, 2010;

Gullón *et al.*, 2017). The extracts showed different degrees of efficiency concerning the conditions in which the samples were extracted. Among the bacterial strains, *Listeria monocytogens* were the most sensitive, and *Pseudomonas aeruginosa* corresponded to the most resistant bacterial species.

According to these authors, considering the results for MIC and MBC tests, Gram-negative bacteria were more resistant than Gram-positive bacteria. This may be due to the layer of lipopolysaccharides present in the membrane of Gram-negative cells, which makes access to the membrane more restricted.

2.4.2. Antifungal activity

The use of natural extracts and essential oils are considered alternatives to overcome the reported problems associated with synthetic fungicides that are normally used to prevent both fungal attacks and mycotoxin contamination. The use of synthetic substances may include side effects, as the residual toxicity that contributes to the development of fungal resistance.

The antifungal activity of natural extracts can be evaluated by inhibiting fungi growth, whether yeast or filamentous, through direct contact of the sample with the strain. Some methods are presented in the literature, with different approaches and principles. Scorzoni *et al.* (2007) attest that the most used tests are bioautography, disk diffusion, agar dilution, and dilution tests. The methods generally may be modified according to specific strains samples and therefore is not recommended to compare the results directly if the method is not correspondent (Scorzoni *et al.*, 2007).

Although the application of plant matrices extracts is a promising strategy, in-depth research in this area is still needed to reach a sufficiently competitive commercial product. According to Barbosa *et al.* (2016), chemical compounds present in *E. globulus* leaves have inhibitory activity against *Candida albicans, Fusarium oxysporum*, *Mucor spp., Penicillium digitatum, Rhizopus nigricans, Rhizopus solani*, *Saccharomyces cerevisiae*, *Trichophyton spp,* and some species of the *Aspergillus genus*, with the lowest inhibitory concentration reported to *C. albicans*.

2.4.3. Antiviral activity

The pharmaceutical industry is increasingly targeting medicinal plants to identify lead compounds with high added value, focusing on alternative antiviral agents of natural origin. Commonly used antiviral medication inhibits DNA polymerases to avoid the spread of viruses in the host but encountering compounds with viral targets is of particular interest to avoid resistance and undesirable side-effects from synthetic drugs.

A published study by Brochot *et al.* (2017) evaluated the antiviral activity of blends composed of extracts of *Eucalyptus globulus* (leaf), *Cinnamomum zeylanicum* (bark), *Rosmarinus officinalis* (leaf), *Daucus carota* (seed), and *Camelina sativa* (seed) that resulted in a significant reduction of viral units for H1N1 and HSV1. Additionally, *E. globulus* main compound 1,8-cineole and beta-caryophyllene exhibit an anti-HSV1 activity by directly inactivating free-virus particles and might interfere with viral envelope structures required for entry into host cells (Astani *et al.*, 2010; Brochot *et al.*, 2017). On the other hand, Cermelli *et al.* (2008) tested two clinical strains (adenovirus and mumps virus) adapted to grow in cells *in vitro*. Results showed a mild antiviral activity against the mumps virus, but not against adenovirus, once again speculating that this low activity might be due to its enveloped configuration, as it also occurs in the case of the herpes simplex virus in Brezáni *et al.* (2018) and Astani *et al.* (2010).

Given the tragic impact of the COVID-19 pandemic, plant biotechnologies with the potential to fight this virus have been developed as useful alternatives for treatment and prophylaxis. In a preliminary study, Sharma & Kaur (2021) used isolated bioactive molecules present in *E. globulus* leaves to report the potential for inhibiting SARS-CoV-2 through molecular docking. The binding affinity between the molecules and proteases of the COVID-19 virus (3CLpro) were evaluated since this binding plays a vital role in their replication. Wu *et al.* (2004) reported activity against SARS-CoV for eucalyptus extract at a concentration of 100 µM using cell-based assay, with SARS virus and Vero E6 cells.

2.4.4. Antioxidant activity

Free radicals can be harmful to cell biomolecules such as nucleic acids, proteins, lipids, and carbohydrates, and contribute to oxidative stress that causes several diseases (Lobo *et al.*, 2010). The antioxidant activity is usually attributed to the presence of phenolic

compounds including hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, and tannins (Boulekbache-Makhlouf *et al.*, 2013), thus antioxidant phenols interfere with the production of free radicals and play a key role in their inactivation. Plant extracts are natural sources of phenolics and can be used as antioxidants vectors considering the bioavailability and bio-effectiveness of this class of compounds (Amakura *et al.*, 2009).

The antioxidant activity (total antioxidant capacity) of plants and plant extracts can be determined by cell-based tests, which can be classified by: evaluating the ability to inhibit lipid peroxidation, by measuring the formation of substances reactive to thiobarbituric acid (TBARS); and evaluating the anti-hemolytic capacity, through the oxidative hemolysis inhibition test (OxHLIA) (Dasgupta & Klein, 2014; Takebayashi *et al.*, 2010). Although obsolete, there are also other general types of tests associated with the elimination of electrons or radicals through reduction reactions: DPPH test (1,1-diphenyl-2-picrylhydrazyl), Trolox equivalent antioxidant capacity parameter (TEAC), and ferric reducing antioxidant power assay (FRAP) (Škrovánková, Mišurcová and Machů 2012). According to Wong *et al.* (2006), more than one type of measurement of antioxidant activity should be performed taking into account the various mechanisms of antioxidant action.

Being one of the oldest antioxidant assays, but still widely used, the TBARS (thiobarbituric acid reactive substances) assay quantifies oxidative stress by measuring peroxidative damage to lipids. This damage occurs due to the presence of free radicals that, in contact with lipids, resulting in loss of selectivity in ion exchange and release of organelle content (*e.g.* hydrolytic enzymes in lysosomes) and formation of cytotoxic products, such as malondialdehyde (MDA), a substance produced due to the degradation of unstable lipid peroxides (Dasgupta & Klein, 2014). In the TBARS assay, MDA reacts with 2-thiobarbituric acid (TBA) under conditions of high temperature and acidity, generating a chromogen that can be measured by spectrophotometric/spectrofluorometric methods (Oakes & Van Der Kraak, 2003). González-Burgos *et al.* (2018) investigated the effect of *E. globulus* extracts on lipid peroxidation using the TBARS assay. SH-SY5Y cells (human neuroblastoma) were pre-treated *in vitro* with the extracts, resulting in a considerable decrease of 151.4% in lipid peroxidation, using ethanolic extract at a concentration of 5 mg of dry weight per mL of solvent.

The OxHLIA assay is based on the ability to inhibit free radical damage to the erythrocyte cell membrane. According to Takebayashi, Chen, and Tai (2010), the advantage of this method is that it uses peroxyl radicals as pro-oxidants and erythrocytes as oxidable targets, thus the results obtained reflect the efficacy of eliminating biologically relevant radicals.

The DPPH method is based on the elimination of DPPH by antioxidants, which discolor the DPPH methanolic solution after a reduction reaction. This test measures the antioxidant reduction capacity concerning the stable radical. It is widely used due to the relatively short time required for analysis (Škrovánková *et al.*, 2012). The TEAC assay is also widely used to measure the total radical scavenging capacity. The test is based on the discoloration of the ABTS radical (2,20-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid]) by antioxidant compounds, thus reflecting the amount of ABTS eliminated in comparison to the elimination of the Trolox (6-hydroxy acid) 2,5,7,8-tetramethylchroman-2-carboxylic). The total radical scavenging capacity of the sample is calculated by relating the decrease in absorbance in solutions with ABST and Trolox at 734 nm. FRAP determination is used to measure the antioxidant power of plant extracts in their ability to reduce Fe^{3+}-tripyridyltriazine to Fe^{2+}-tripyridyltriazine. The assay is based on electron transfer reactions in which a ferric salt (potassium ferricyanide is used as an oxidizer) and absorbance measurement at 593 nm (Škrovánková *et al.*, 2012).

Gullón *et al.*, (2017) conducted antioxidant capacity tests evaluating optimal conditions of temperature, ethanol concentration (solvent), and time. The DPPH assay was done by adding 2 mL of a methanolic solution of DPPH (6.10^{-5} M) to 0.2 mL of an ethanolic solution of the extract. The decrease in absorbance at 515 nm was recorded after 16 min. The Trolox equivalent antioxidant capacity (TEAC) was measured using 2 mL of diluted $ABTS^{\bullet+}$ (radical cation of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) solution to 20 μL of diluted extracts. The authors also performed the FRAP test mixing the diluted extracts (0.1 mL) with 3 mL of the FRAP reagent. The absorbance was recorded after 6 min at 593 nm. According to the authors, temperature and ethanol concentration in the solvent are the most influential variables, and the extract displayed strong antioxidant activity.

2.4.5. Cytotoxic activity

Some authors have reported the cytotoxic antitumor bias of extracts from parts of *E. globulus* with proven competitive efficacy compared to synthetic assets used in the treatment of tumor cells. Adnan (2019), for example, evaluated the cytotoxic effect of aqueous extracts against lung cancer cells (A549 cell line) and concluded that it is possible to incorporate the extract into the treatment, currently done with the chemotherapy drug Fluorouracil (5FU).

In Teixeira *et al.* (2019) the inhibitory effect of cell growth of *E. globulus*, prepared by decoction and infusion in distilled water, was tested in three models of human tumor lines: lung cancer (NCI-H460), colorectal adenocarcinoma (HCT- 15), and pancreatic cancer (PANC-1). Both extracts inhibited the growth of all cell lines and the decoction extract was the most potent in inhibiting the growth of NCI-H460 cells.

In another study, by Ghareeb *et al.* (2019), the hepato-renal protective activities of *E. globulus* were examined on liver and kidney tissue of mice and compared with drugs commonly used in the treatment of hemorrhagic cystitis, in which the extract showed successful applicability in the treatment of this disease and other similar illnesses.

In traditional doses (1.5–3 g of dried leaves in 150 ml, up to four times daily) (EMA, 2013), there is no report on the toxicity of *E. globulus*. However, overdoses doses may cause nausea, vomiting, and diarrhea (World Health Organization, 2002). Overall, its safety is classified as high, according to Silveira *et al.* (2020).

2.5. Extraction of bioactives using green solvents

The toxicity of solvent residues in target compounds coupled with low extraction yield has stimulated interest in developing environment-friendly extraction methods, focusing on minimizing or eliminating the use of volatile toxic organic solvents (Bubalo *et al.*, 2018). Commercial interest in more sustainable, non-toxic routes of extraction has increased, driven by growing consumer demands for greener alternatives and natural ingredients that do not involve toxic chemicals and the environmental and health risks associated with the use of chemical solvents. A major challenge involving extracting molecules from a complex matrix is that these molecules are usually embedded within the matrix. While there is undoubtedly a clear need to develop affordable, safe, effective, and sustainable extraction techniques, it is important that such techniques not only enable clean label status but also ensure enhanced

yields with minimal impact on the quality of the end product (Bubalo *et al.*, 2018; Choi & Verpoorte, 2019; Pena-Pereira & Tobiszewski, 2017).

Despite the wide range of scientific and technological applications, some traditional volatile organic solvents can be ascribed to many problems concerning safety risks. Halogenated and aromatic hydrocarbon solvents, for example, were willingly applied because of their excellent properties to dissolve many organic compounds, although they present high oral and inhalation toxicities and some other chronic negative effects, as teratogenicity and carcinogenicity. Because of their high volatility in many cases, the exposure for long periods can lead to acute and chronic threats. In addition, a wide range of organic solvents remains can persist in the environment, causing direct bioaccumulation and ozone depletion, among other complications (Pena-Pereira and Tobiszewski 2017).

Regulations have been implemented to decrease the consumption of solvents of very high concern and harmful to human health and the environment. Regulation (EC) No 1907/2016 established the phaseouts for reducing solvents classified as carcinogenic, mutagenic, or toxic to reproduction for developed and developing countries under the Montreal Protocol. Unfortunately, hazardous organic solvents are widely applied in many industrial processes, thus contributing to the generation of significant amounts of residues. The development of greener alternatives is crucial indeed, bearing in mind the environmental, health, and safety risks attached to the use of these substances (Pena-Pereira & Tobiszewski, 2017).

Conventional extraction techniques for plant bioactive compounds are usually associated with a high volatile organic solvent consumption and long extraction times. To establish an environmentally friendly extraction and separation method for bioactive compounds, ionic liquids and eutectic solvents have been studied as novel green alternatives to conventional solvents (Li & Row, 2016).

Ionic liquids are defined as salt mixtures in which compounds bind to each other through ionic bonds, with melting points inferior to 100 °C. Their characteristic physicochemical properties are distinguished from conventional organic solvents due to their low vapor pressure, high thermal stability, and conductivity, for example, being, therefore, a viable alternative to conventional solvents in various chemical processes such as extractions, chemical syntheses, and enzymatic reactions (Choi & Verpoorte, 2019).

However, given the toxicity, high-cost synthesis, and low rate of degradation in the environment when compared to other green solvents have driven the development of other alternative types, *i.g.* deep eutectic solvents (DES).

Deep eutectic solvents are defined as combinations of two or three components forming a eutectic mixture with a melting point below the values for pure compounds (Sunol *et al.*, 2019). The first studies involving DES were carried out by Abbott *et al.* (2003), in which the author described the formation of a eutectic mixture from two solid materials with a high melting point, choline chloride and urea, in the proportion of 1:2 at which the melting point became 12 °C (Abbott *et al.*, 2002).

DESs have demonstrated unique physicochemical properties, as low vapor pressure and non-flammability, besides allowing the design according to the desired properties, and have also become a topic of growing interest to both research and industry because they have similar physical and chemical properties, but are cheaper, safer, and easier to obtain than ionic liquids (Li & Row, 2016).

In their reviewing work, Li and Row (2016) refer to some studies regarding the use of DES in the extraction and separation of bioactive compounds from plants, in which most of them presented great efficiency for several species. Additionally, Gullón *et al.* (2019) characterized the extraction of *E. globulus* contents using natural deep eutectic solvents using four types of DES, being three of them based on choline chloride as hydrogen bond acceptor, and ethylene glycol (ChE), xylitol (ChX) or glucose (ChGlu) as hydrogen bond donors. The fourth solvent tested was composed of citric acid and glucose (CaGlu). Powdered leaves were macerated at 50 °C for 60 minutes. Despite the overall substantially lower extraction capacity of DES in this study, chlorine chloride-ethylene glycol (ChE) presented a competitive response in obtaining phenolic and flavonoids compounds, but inferior capacity concerning antioxidants in comparison to other organic solvents. The authors support complementary studies evaluating other different DES families to achieve better extraction results.

2.6. Extraction modeling and optimization

Experimental models address the influence of process variables on specific responses, ensuring reliable results and consistent statistical values. When applied with optimization methods, theoretical models can reduce the number of experiments or repetitions and

improve the quality of results, thus reducing work, time and consequently cost (Barros Neto *et al.*, 2010).

For that reason, modifications are made to the control variables of the system by observing the output data. Afterward, inferences are made concerning which variables are accountable for the responses obtained (Montgomery, 2012). A common obstacle in this type of investigation is determining the influence of one variable on another of interest. In other words, the system acts as a mathematical function, initially unknown, that operates on the input variables (factors) and produces the observed responses (Barros Neto *et al.*, 2010). Therefore, the objective for this work stage and most experimental designs is the definition, or at least a satisfactory approximation, of a function that could represent the behavior of the studied system.

The goal of optimization in plant extracts is to determine the optimal extraction conditions that provide the highest content of target compounds and, in some cases, the highest bioactivity at the same time. Some authors evaluated the extraction yield, however, the optimal yield conditions for yield do not return maximum values for phenolic compounds recovery (Gullón *et al.*, 2017; I. Mota *et al.*, 2012; Rodrigues *et al.*, 2018a). In general, studies reported in the literature on extraction optimization use the Response Surface Methodology (RSM) to find the most suitable model, coupled with numerical optimization methods that allow estimating the conditions to produce an extract with relative precision.

Response Surface Methodology (RSM) aims to model, analyze, and optimize systems in which a response of interest (y) is influenced by several variables ($x_1, x_2, \ldots, x_n$). It normally represents the response surface graphically (Figure 2.7.a), on which the response is plotted according to the levels of the variables (Montgomery, 2012). For visual comprehension, RSM models usually have contour curves, where each curve corresponds to a response level (Figure 2.7.b). In most RSM problems, the shape of the relationship between the response and the independent variables is unknown. Thus, the first step is to find an appropriate mathematical function as close as possible to the real (Montgomery, 2012).

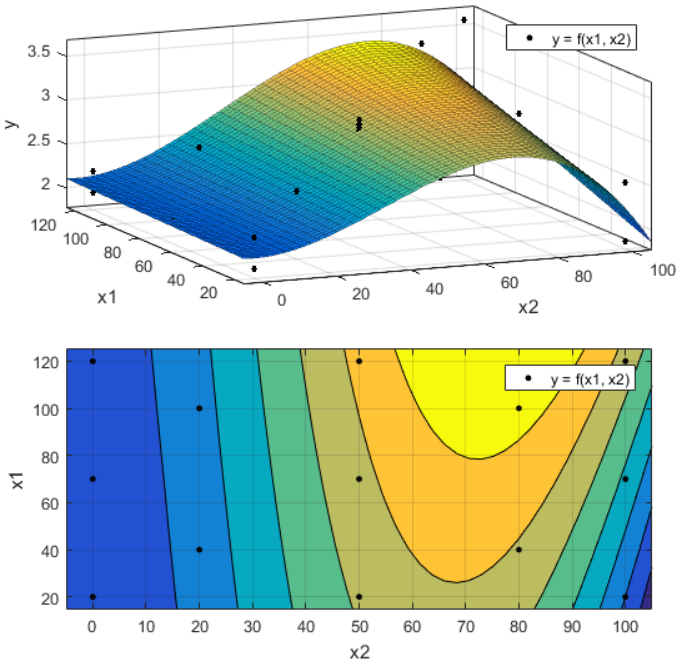


Figure 2.7: A three-dimensional response surface of the response (y) as a function of two variables (x_1 and x_2) (a) and a contour plot of a response surface (b).

A polynomial model is unlikely to be a great approximation of the function across the space of the independent variables, but for a limited region and of experimental interest as in the case of plant extractions, they generally work very well (Cornell, 1992; Montgomery, 2012).

Assuming the experimental design is represented by a regression model, where an observed response $y(x_1, x_2, \ldots, x_k)$ is considered a random variable which is distributed around a population mean $\eta(x_1, x_2, \ldots, x_k)$ with a population variance σ^2 (Barros Neto *et al.*, 2010):

$$y(x_1, x_2, \ldots, x_k) = \eta(x_1, x_2, \ldots, x_k) + \epsilon(x_1, x_2, \ldots, x_k) \quad (2.6.1)$$

being ϵ the random error of responses concerning the population mean and can be calculated by the difference between the observed response (y_i) and the response predicted by the model ($\hat{y}_i$), as can be seen at (Eq. 2.6.2). Errors are normal and independently distributed, considering that the mean of the errors is equal to zero and σ2 is constant.

$$\epsilon_i = y_i - \hat{y}_i \quad (2.6.2)$$

The mean of the population η can be adequately described by the model represented in Eq. 2.6.3:

$$\eta(x_1, x_2, \dots, x_k) = \beta_0 + \sum_{i=1}^{n} \beta_i x_{ik} \tag{2.6.3}$$

where β_0 corresponds to the linear regression coefficient, and β_i represents the coefficients concerning the interactive effects among the factors evaluated (Montgomery, 2012, p. 451).

Least square method (LSM) is usually applied to estimate the parameters in the approximation polynomials. From LSM polynomial coupled with RSM original surface, an adjusted surface is built, representing an adequate approximation of the real response function.

The adjustment by the regression analysis imposes that the equation defined by the coefficients β must converge as possible to the observed responses y, that is, the vector of residuals ϵ should be close to zero. The residuals (Eq. 2.6.2) must be squared to always present positive values and the sum of the squared residuals *L* (least square function) must be as much close to zero as possible for an admissible regression model (analytical and matrix notation of L in Eq. 2.6.4 and Eq. 2.6.6, respectively) (Montgomery, 2012, p. 451).

$$L = \sum_{i=1}^{n} \epsilon_i^2 = \sum_{i=1}^{n} \left(y_i - \beta_0 - \sum_{j=1}^{k} \beta_j x_{ij} \right)^2 \tag{2.6.4}$$

Rencher and Schaalje (2008) suggest the determination of the vector of the least-squares estimators that minimizes *L*, and the equalization of its derivative with respect to β_0. For the function *L* to be minimized with respect to $\beta_1, \beta_2, \dots, \beta_k$, these least square estimators must satisfy the relation presented in Eq. 2.6.5:

$$\left. \frac{\partial L}{\partial \beta_0} \right|_{\hat{\beta}_1, \hat{\beta}_2, \dots, \hat{\beta}_k} = -2 \sum_{j=1}^{n} \left(y_i - \hat{\beta}_0 - \sum_{j=1}^{k} \hat{\beta}_j x_{ij} \right) = 0 \tag{2.6.5}$$

The two analytical expressions previously described can be simply expressed in matrix notation:

$$L = y'y - 2\beta' X' y + \beta' X' X \beta \tag{2.6.6}$$

$$\left.\frac{\partial L}{\partial \beta}\right|_{\hat{\beta}} = -2X'y + 2X'X\hat{\beta} = 0 \tag{2.6.7}$$

where X' is the transpose matrix of X. Equation 2.6.7 can be simplified to obtain the matrix of the least square estimators ($\hat{\beta}$):

$$\hat{\beta} = (X'X)^{-1}X'y \tag{2.6.8}$$

It is important to note that, not only the estimators $\hat{\beta}_i$ and the L function can be described in terms of observations under the matrix notation:

$$\hat{y} = X\hat{\beta} \tag{2.6.9}$$

where

$$y = \begin{bmatrix} y_1 \\ y_2 \\ \vdots \\ y_n \end{bmatrix}, \quad X = \begin{bmatrix} 1 & x_{11} & x_{12} & \cdots & x_{1k} \\ 1 & x_{21} & x_{22} & \cdots & x_{2k} \\ \vdots & \vdots & \vdots & & \vdots \\ 1 & x_{n1} & x_{n2} & \cdots & x_{nk} \end{bmatrix}, \quad \beta = \begin{bmatrix} \beta_1 \\ \beta_2 \\ \vdots \\ \beta_n \end{bmatrix}, \quad \epsilon = \begin{bmatrix} \epsilon_1 \\ \epsilon_2 \\ \vdots \\ \epsilon_n \end{bmatrix}$$

Additionally, the standard error (SE) of $\hat{\beta}_i$ may be obtained through:

$$SE(\hat{\beta}_i) = \sqrt{\hat{\sigma}^2 C_i} \tag{2.6.10}$$

in which C is the matrix of the diagonal elements of $(X'X)^{-1}$ corresponding to $\hat{\beta}_i$, and $\hat{\sigma}^2$ corresponds to the estimator of the variance and calculated by:

$$\hat{\sigma}^2 = \frac{SS_E}{n-p} \tag{2.6.11}$$

being SS_E the residual sum of squares, p the number of regression coefficients (β_i) and n the number of observations. The parameter $\hat{\sigma}^2$ is useful for the verification of the precision and the statistical relevance of these coefficients, as the smaller is the $SE(\beta_i)$, more accurate is the regression model regarding the coefficient β_i (Montgomery, 2012).

In order to improve the effectiveness and make the model more compact, hypothesis tests are often performed to determine the significance of each coefficient in the regression model. According to (Montgomery, 2012, p. 464), the hypotheses for testing the significance of any individual regression coefficient are:

$$H_0: \beta_j = 0$$

$$H_1: \beta_j \neq 0$$

If H_0 is not rejected, then this indicates that the coefficient in question (β_j) can be excluded from the model. The test statistic for this hypothesis is:

$$t_0 = \frac{\beta_j}{\sqrt{\hat{\sigma}^2 C_{jj}}} \tag{2.6.12}$$

where C_{jj} is the diagonal element of matrix $(X'X)^{-1}$ corresponding to β_j. The null hypothesis is rejected when $|t_0| > t_{\alpha/2,\ (n-k-1)}$.

3. Materials and methods

3.1. Plant Material

Eucalyptus (*E. globulus*) leaves were collected near Águeda, in the center western region of Portugal, in October 2020. The samples were air dried and cut into pieces, then reduced to 20 mesh to guarantee sample homogeneity, and stored in an airtight flask, protected from light and humidity until further analysis.

Figure 3.1: Grounded *E. globulus* leaves (20 mesh).

3.2. Experimental design, modeling, and optimization

Response surface methodology is often used when it comes to solving optimization problems due to its applicability and suitability to different cases. In this work, a self-authoring algorithm was developed as an alternative to traditional methods, called Dynamic RSM. This proposed method was based on the learn machine principle, using the genetic algorithm combined with clustering and bootstrap analysis. The performance and robustness of dynamic RSM were evaluated according to the results obtained, analysis of variance, correlation with data obtained experimentally and via Traditional RSM.

The two constrained optimization algorithm (Traditional RSM, previously described in Section 2.6, and Dynamic RSM) were developed in the MATLAB® computing platform (MathWorks, Inc., R2009a 9.6 version), based on Response Surface Methodology (RSM) and Central Composite Circumscribed Design (CCCD), to optimize the HAE and UAE conditions for the extraction of phenolic compounds and total yield from *E. globulus*. The coded and natural values of the independent variables x_1 (time, t in min), x_2 (temperature, T

in °C), and x_3 (solvent, *S* in % of ethanol, v/v) are presented in Table 3.1 for HAE and UAE. Responses were achieved using 22 independent combinations and 6 repetitions at the center of the experimental domain, totaling 28 experimental points.

In this work, the points were generated in a sphere around the central point (Figure 3.2), with five levels of each factor (-1.68 to 1.68). The central point supposedly coincides with the optimal combination for the response, thus it was repeated several times to maximize the prediction (Box *et al.*, 2005). To minimize the effects of unexpected variability in the observed responses, the experimental runs were randomized.

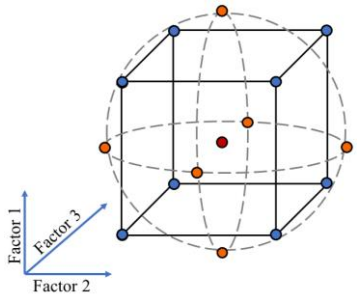


Figure 3.2: Example of spherical CCCD of three factors and five levels (adapted from Montgomery 2012).

From the obtained responses, considering the combinations proposed by the CCCD, the coordinates were adjusted employing the least-squares analysis with the following second-order multivariate model (Montgomery, 2012, p. 479):

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{\substack{i=j \\ j>1}}^{k-1} \sum_{j=2}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} x_i^2 \qquad (3.2.1)$$

Y corresponds to the predicted response values (described) for a given composition of variables *x*; x_i and x_j are the independent variables used during the extraction (the conditions of *t*, *T*, and *S*); b_0 is the constant term; b_i is the linear effect factor; b_{ij} is the factor of the interaction effect between two variables, b_{ii} is the factor of the individual quadratic effect and *k* is the number of variables.

3.2.1. Traditional RSM

The algorithm based on the traditional method starts by using the points generated from the CCCD to develop a multivariate function (objective function), which represents the extraction behavior in terms of the studied responses. The function is maximized from the Newton-Raphson numerical method, iteratively, until an approximation by convergence is reached, according to the established error tolerance. The iterative process is presented in Figure 3.3.

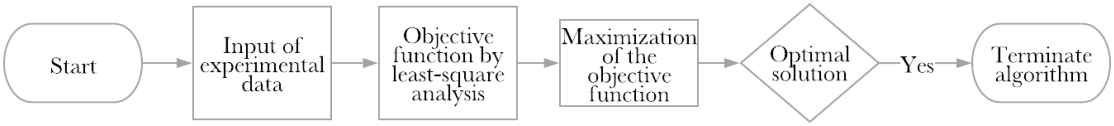


Figure 3.3: Flowchart of a traditional RSM modeling approach for optimal design.

3.2.2. Dynamic RSM

The dynamic method is also based on CCCD points, however, it uses machine learning-based steps, such as the genetic algorithm (GA), clustering, and bootstrap analysis for the arrangement of the sample dataset.

The genetic algorithm is based on the evolutionary process of natural selection and genetic dynamics that seeks the ideal combination of parameters with an exchange of random but structured information to estimate an optimal solution (El-Mihoub *et al.*, 2006). In this manner, in each iteration, a unique search path was built, as new solutions were obtained and added to the set of previous solutions. Since constrained optimization problems can influence the sampling capacity of a genetic algorithm due to the population limits considered, the incorporation of a local optimization method combined with GA was implemented to minimize drift errors.

Cluster algorithms were introduced in the code to categorize the combinations generated by GA into subdomains according to their pattern to assess the performance of the method. The means and the medoids of the clusters were considered for each studied answer. Bootstrap analysis was applied to evaluate the variability of the optimal solutions derived from the analysis of the clustering method, resampling from the original sample data set. Thus, the confidence interval of the statistic of interest was estimated by comparing the

results obtained by traditional methods. Figure 3.4 summarizes the steps of the dynamic response surface method.

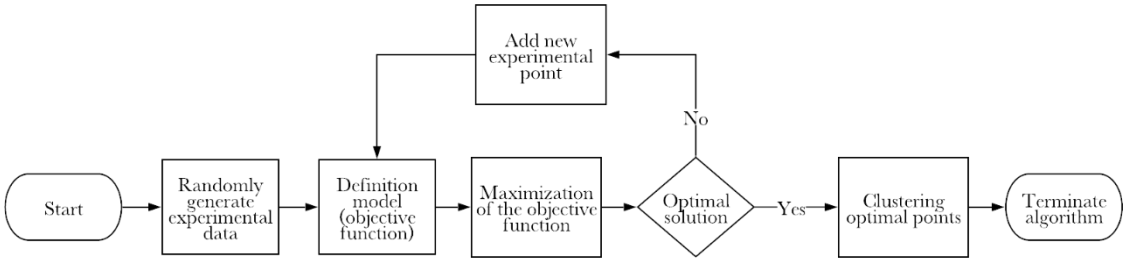


Figure 3.4: Flowchart of the Dynamic RSM integrating Genetic Algorithm and cluster analysis to the process.

The Dynamic RSM was developed in this work, aligned with pre-existing functions of the statistical and optimization toolboxes of the software. The algorithm starts by generating a set of 15 random combinations from the levels established in Table 3.1. From this set of initial data, a multivariate regression model is calculated, this model being the objective function of the problem. After that, an integrated Genetic Algorithm solver was used to estimate the optimization problem. When the optimal combination is identified, it is added to the data population and a new objective function is calculated based on this new data set. The iterative process stops when the last optimal solution has a minimum tolerance limit. Considering the stochastic nature of this case study, cluster analysis was used to evaluate the precision of the algorithm based on the closest coordinate to the ideal solution. For this analysis, 100 runs were carried out to evaluate the effectiveness of the method, divided into 3 groups by clustering analysis according to the patterns identified considering all runs. The combinations of parameters that occur most often in the collection were determined as optimal extraction conditions. To handle the variability of the optimum achieved, the bootstrap method is used to estimate the 95% confidence interval.

The general objective of using programming tools for the optimization of this type of study is to minimize the number of experiments, avoid time-consuming calculations and facilitate the understanding of the most influential variables for the extraction. Based on the variables coded according to the CCCD, the developed algorithm transformed the problem (an unconstrained multivariable function) into a subproblem, easier to solve and which is

used as the basis of an iterative process, until the maximum response value is found, belonging to the domain limited by the restrictions.

3.3. Extraction methods

Based on a combination of previous plant extractions performed at CIMO – Centro de Investigação de Montanha (Mountain Research Center) – and previous extractions studies found in the bibliographic material available (Table 2.1), the relevant variables and appropriate ranges for the studied extraction techniques (HAE and UAE) were selected and tested. Thus, a detailed description of the variables and considered ranges to support the response surface design is presented in Table 3.1. The particle size (20 mesh) and solid-liquid ratio was kept constant (30 g/L) for both extractions, based on previous studies performed by Gullón *et al.* (2019) The applied solvent was a mixture of ethanol/water, characterized in terms of ethanol content (%, v/v). In this work, ethanol was chosen for being an organic solvent commonly used for extraction of plant matrices due to its high extractive power and lower toxicity in comparison to other solvents also widely used, such as methanol or dichloromethane. All experimental points were extracted and analyzed in duplicate.

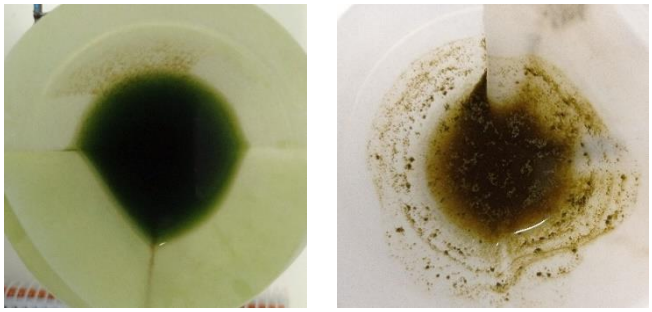
Table 3.1: Experimental domain and codification of independent variables in the CCCD factorial design with 5 range levels.

Coded values	Natural values					
	Heat-assisted extraction			Ultrasound-assisted extraction		
	t (min)	T (ºC)	S (%)	t (min)	P (W)	S (%)
-1.68	20	25	0	3	100	0
-1	40	37	20	11.5	180	20
0	70	55	50	24	300	50
+1	100	73	80	36.5	420	80
+1.68	120	85	100	45	500	100

3.3.1. Heat-assisted extraction (HAE)

Heat-assisted extraction (maceration) was performed by adding the dried powdered leaves (1.5 g) and 50 mL of solvent to a sealed amber flask, to avoid solvent loss and possible interference of light. The vessel was inserted into a thermostatic water bath under continuous electromagnetic stirring (CIMAREC Magnetic Stirrer with a fixed agitation speed of 500 rpm; Thermo Scientific, San Jose, CA, USA) under the required conditions of the work plan (t, T, and S) presented in Table 3.1. After extraction, the mixture was filtered through

Whatman filter paper No. 1, and the filtrate was stored in a sealed falcon under refrigeration (-4 °C) and protected from light.



(a) (b)

Figure 3.5: Crude extract of Eucalyptus leaves using pure ethanol (a) and water (b). The green pigment of the ethanolic extract is probably due to the presence of chlorophyll, which is very soluble in ethanol and insoluble in water (absence of a greenish color in aqueous extract).

The experimental procedure for the optimal points was performed by adding the dried powdered leaves of *E. globulus* to a pre-heated solvent (SLR of 30 g/L) to a sealed flask to avoid solvent loss. The flask was connected to a Carousel 6 Plus Reaction Station™ (Radleys Tech, Essex, UK) under agitation and coupled with a thermometer to control the temperature during extraction. This equipment has better control of the variables involved, which is an intrinsic factor in the acquisition of accurate data for the optimization of the process. For a primary analysis, it is more suitable to achieve the experimental points in the traditional hot water bath since this method is indicated for procedures with high repeatability.

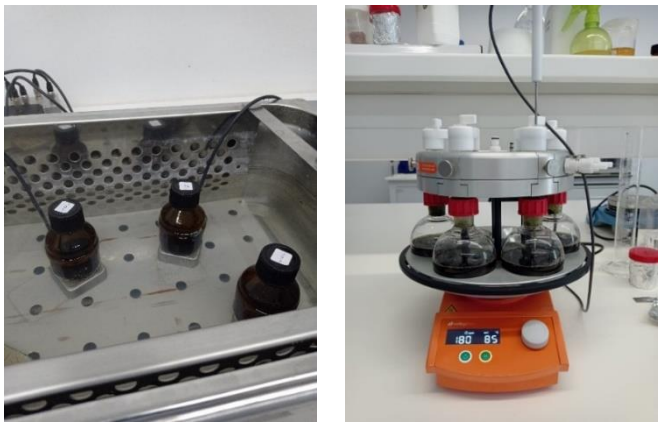


Figure 3.6: HAE extraction of *E. globulus* leaves performed in a hot water bath (left) and a Carousel 6 Reaction Station coupled with a hot stirring plate (right).

3.3.1. Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction (UAE) was performed using ultrasonic equipment (Ultrasonic homogenizer, model CY-500, Optic. Ivymen System, Barcelona, Spain). The dry powdered samples (1.5 g) and 50 mL of solvent were placed in a beaker coupled with an ultrasonic probe and the extraction proceeded under the required conditions of the work plan (t, P, and S) presented in Table 3.1.

In this study, the probe system was chosen due to its higher ultrasonic intensity delivered through a smaller surface (the tip of the probe) in comparison to a conventional ultrasonic bath. By applying punctual power, direct delivery of ultrasound to the extraction medium is achieved with minimal loss of ultrasonic energy (Chemat *et al.*, 2017). The solid/liquid ratio (30 g/L) and the temperature were kept constant during extraction by uninterrupted refrigeration of the system to avoid excessive heating, using a thermometer for temperature control and verification. After the extractions, the mixtures were filtered through Whatman filter paper No. 1, and the filtrates were stored in falcon tubes.

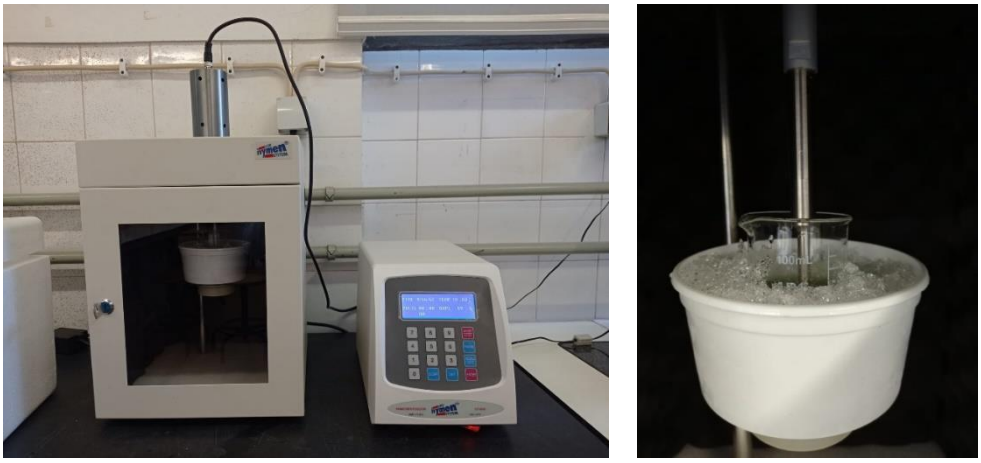


Figure 3.7: Ultrasound device (left) and ultrasound-assisted extraction being performed using an attached probe (right).

3.4. Determination of extraction yield

The filtrate resulting from each extraction was determined gravimetrically. From each filtrate, 5 ml was transferred to a small crucible and heat treatment in a laboratory oven at 100 °C for about 72 hours to determine the dry weight of the extracts after evaporation of the solvent. The results were expressed as mg/g of dry weight of leaves (dw).

3.5. Chromatographic identification and quantification of phenolic compounds

Each experimental point was filtered through a disposable LC filter disk (0.22 µm) before chromatographic analysis, which was performed with an HPLC-DAD-ESI/MS[2] (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) system. Detection was carried out by diode array detection (DAD), using 280 and 370 nm as the preferred wavelengths, coupled to a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source and working in the negative mode. Chromatographic separation was performed using a Waters Spherisorb S3 ODS-2 C18 column (3 µm, 4.6 mm x 150 mm, Waters, Milford, MA, USA) at 35 °C, using acid/water (0.1%) and acetonitrile as mobile phase. Data acquisition was performed using Xcalibur data system (Thermo Finnigan, San Jose, CA, USA).

Phenolic compounds were identified considering their retention times, UV-*vis* spectra, and mass spectrum, with comparison to commercial standards and the available literature data. In the case of commercially unavailable standards, the compounds were quantified using a calibration curve of the most similar compound available. The results were expressed in mg/g of dry weight (extract) using the following standards: *p*-hydroxybenzoic ($y = 18424x – 109642$; $R^2 = 0.9984$) and caffeic acids ($y = 47221x - 228640$; $R^2 = 0.9989$) for phenolic acids, and quercetin 3-*O*-glucoside ($y = 34\ 843x - 160\ 173$; $R^2 = 0.999$) for flavonoids.

3.6. Bioactivity assays

The bioactivity tests were carried out considering the four optimum points achieved in the optimization of the extraction: Total Yield (Y), Total Phenolic Acids Content (TPA), Total Flavonoids Content (TFC), and Total Phenolic Content (TPC). The ethanol present in each optimum point was evaporated at 40 ºC using a vacuum rotary evaporator (Büchi, R-210, Flawil, Switzerland). Therefore, the aqueous fraction was lyophilized (47 ºC, 0.100 bar; FreeZone 4.5, Labconco, Kansas City, MO, USA) to obtain the powdered extract. The extracts were stored away from moisture and light until further analysis.

[2] High performance liquid chromatography equipped with diode array detection and electrospray ionization mass spectrometry.

3.6.1. Antioxidant activity

The antioxidant activity was analyzed through two assays, one, using porcine brain tissue to mimic high-fat cells (TBARS), and another with murine macrophage cells (cellular antioxidant activity). The use of more than one antioxidant assay relies on the fact that oxidation and antioxidants have several processes through which they can take place, and thus, using several assays allows understanding the antioxidant activity of the sample through several of these mechanisms.

The antioxidant activity was first analyzed concerning lipid peroxidation through Thiobarbituric Acid Reactive Substances (TBARS) assay. Briefly, cellular lysates were prepared by adding porcine brain cells to Tris-HCl buffer solution (20 mM, pH=7.4, refrigerate), then centrifuging the suspension at 3500 g for 10 min. The lysate samples were incubated with thiobarbituric acid (TBA), trichloroacetic acid (TCA), and HCl (hydrochloric acid) reagent in water bath at 37.5 °C for 10 min. 10 mg of each extract was added to 1.0 mL of Tris-HCl, from which successive dilutions were carried out, obtaining the concentrations to be tested. 100 uL of ascorbic acid, 100 uL of iron sulfate and 100 uL of the lysate suspension were added to each extract dilution. Two control samples were prepared with Tris-HCl buffer solution and with an ethanolic solvent correspondent to the optimal for HAE and UAE. The dilutions were placed in a water bath for 20 min at 80 °C. Absorbance was measured at 535 nm in a microplate reader (Bio-Tek Instruments, Inc.; Winooski, USA). The antioxidant activity was expressed in EC_{50} (the concentration that reduces by 50% the oxidants existing in the solution).

The second test evaluated the cellular antioxidant activity (CAA), followed as described by (Wolfe & Rui, 2007). The extracts were dissolved in H_2O to obtain a concentration of 8 mg/mL, from which successive dilutions were made 2',7'-dichlorodihydrofluorescin (DCFH) prepared with ethanol and diluted with Hanks' Balanced Salt Solution (HBSS, 50 μM), obtaining the concentrations to be tested (500 - 2000 μM). The cell line RAW 246.7 (murine macrophage cells), obtained from DMSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, was maintained in an incubator at 37 °C, with a humidified atmosphere and 5% CO_2 and DMEM culture medium supplemented with L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), fetal serum bovine (10%) and non-essential amino acids (2 mM).

Murine macrophages were detached with a cell scraper and the content was transferred to a falcon. The solution was centrifuged for 5 minutes at 1200 rpm. The medium was discarded, and the amount of new medium was added according to the size of the obtained pellet. A solution with a cell density of 70.000 cells/mL was then prepared. An aliquot of the prepared solution (300 μL) was transferred to black microplates with clear-bottom (SPL Lifesciences, Gyeonggi, Korea) and incubated for 48h. After the incubation period, the medium was discarded and the cells were washed with HBSS (2x, 100 μL), treated with different extract concentrations (200 μL, 32.5 - 2000 μM), and incubated for 1 hour. Afterward, the cells were washed with HBSS (2x, 100 μL) and added to a solution of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH, 100 μL, 600 μM). Fluorescence was read every 5 minutes for 1 hour (Biotek ELx800 microplate reader, Bio-Tek Instruments, Inc., Vermont, US) at 485 nm excitation and 538 nm emission. Quercetin was used as a positive control, and DCFH and DMEM culture medium were used as a negative control.

3.6.2. Antimicrobial activity

Antibacterial activity

The antibacterial activity was evaluated through the microdilution method. Samples of 100 mg were prepared and analyzed to obtain the minimum inhibitory concentration (MIC) which is known as the lowest concentration that inhibits visible bacterial growth, and minimum bactericidal concentrations (MBC) which present the lowest concentration that effectively eliminates bacteria. The extracts were tested against some food contaminants, five Gram-negative bacteria, namely, *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610), and three Gram-positive bacteria, namely *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 25923). All these microorganisms were purchased from Frilabo, Porto, Portugal. The bacteria were incubated at 37 ºC an appropriate fresh medium, for 24 h before analysis to maintain the exponential growth phase.

The MIC determinations on all bacteria were conducted using colorimetric assay according to described by Pires *et al.* (2018). The samples were first dissolved in 5% (v/v) dimethyl sulfoxide and 95% of autoclaved distilled water to obtain a final concentration of 20 mg/mL for the stock solution. 90 μL of this concentration was added in the first well (96-

well microplate) in duplicate with 100 μL of Tryptic Soy Broth (TSB). In the remaining wells, 90 μL of TSB medium were added. Then the samples were serially diluted to obtain the concentration ranges (10 to 0.03125 mg/mL). To finish, 10 μL of inoculum (standardized at 1.5×10^6 Colony Forming Unit (CFU) /mL) was added at all the well assuring the presence of 1.5×10^5 CFU.

Two negative controls (with TSB and another with the extract) and two positive controls (with TSB and each inoculum, and another with medium, antibiotics, and bacteria) were prepared. Ampicillin and Streptomycin were used for all bacteria tested and Methicillin was used only for *Staphylococcus aureus*. The microplates were incubated at 37 °C for 24 h. The MIC of samples was detected by following addition (40 μl) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride and incubation at 37 °C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determinate by changing the coloration from yellow to pink if the microorganisms are viable. For the determination of MBC, 10 μL of liquid from each well that showed no change in color was plated on solid medium, Blood agar (7% sheep blood), and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth determines the MBC, which is defined as the lowest concentration required to kill bacteria.

Antifungal activity

The antifungal activity was performed according to described by Heleno *et al.* (2013). *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404) were used, being both organisms obtained from Frilabo, Porto, Portugal. The micromycetes were maintained on malt agar, stored at 4 °C, and were further placed in a new medium incubated at 25ºC for 72h. To investigate the antifungal activity, the fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. The samples were dissolved in a mixture of 5% (v/v) dimethyl sulfoxide and 95% of autoclaved distilled water to give a final concentration of 20 mg/ mL for the stock solution. Afterward, 90 μL of this concentration was added in the first well (96-well microplate) in duplicate with 100 μL of Malt Extract Broth (MEB).

In the remaining wells, 90 μL of medium MEB were placed. Then the samples were serially diluted to obtain the concentration ranges (10 to 0.03125 mg/mL). Minimum

inhibitory concentration (MIC) determinations were performed by a serial dilution technique using a 96-well microplate. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentration (MFC) was determined by serial sub cultivation of a 2 μL of tested compounds dissolved in medium and inoculated for 72 h, into microplates containing 100 μL of MEB per well and further incubation 72 h at 26 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial fungicide ketoconazole (Frilabo, Porto, Portugal), was used as positive control.

3.6.3. Cytotoxicity

The evaluation of the cytotoxicity of the optimized extracts was performed using the Sulforhodamine B (SRB) assay, previously described by Barros *et al.* (2013). Vero (African green monkey kidney) was used. The cell line tested was maintained in DMEM medium supplemented with fetal bovine serum (10%), glutamine, and antibiotics. The culture flask was incubated in an incubator at 37 ºC and with 5% CO_2, under a humid atmosphere. The cells were used only when they had 70 to 80% confluence.

The extracts were dissolved in water to obtain the stock solutions with a concentration of 8 mg/mL, from which successive dilutions were made, obtaining the concentrations to be tested (0.125 - 8 mg/mL). 10 μL of each extract concentrations were incubated with the cell suspension (190 μL) in 96-well microplates for 72 hours. The microplates were incubated at 37ºC and with 5% CO_2, in a humid atmosphere, after checking the adherence of the cells. The cell line was tested at a concentration of $1.9x10^4$ cells per well.

After the incubation period, the cells were corrected with trichloroacetic acid (10% w/v; 100 μL) was previously cooled and plates were incubated for 1 hour at 4 ºC, washed with water and, after drying, an Sulforhodamine B (SRB) solution (0.057%, m/v; 100 μL) was added and stored at room temperature for 30 minutes. To remove non-adhered SRB cells, plates were washed three times with a solution of acetic acid (1% v/v) and placed to dry. Finally, adhered SRB cells were suspended with Tris (10 mM, 200 μL) and the absorbance at a wavelength of 540 nm was read in a microplate reader Biotek ELx800 (Biotek Instruments, Vermont, United States). The results are expressed in terms of the

concentration of extract with the ability to inhibit cell growth by 50% - GI_{50} (Barros *et al.*, 2013). As a positive control of the use of ellipticine.

3.6.4. Anti-inflammatory activity

For the evaluation of anti-inflammatory activity, the optimized extracts were tested in macrophage cells RAW 264.7 (mouse macrophage cell line) according to Jabeur *et al.* (2016). Cell cultures were made in DMEM medium, supplemented with 10% heat-inactivated bovine serum and L-glutamine, at 37 °C with 5% CO_2, in humidified air.

The extracts were first dissolved in sterilized water to obtain a final concentration of 8 mg/mL, from which successive dilutions were carried out, obtaining the concentrations to be tested (0.125 - 8 mg/mL). RAW 264.7 (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), was grown in DMEM medium, supplemented with heat-inactivated fetal bovine serum (10%), glutamine and antibiotics, and kept in an incubator at 37 ºC, with 5% CO_2 and under humid atmosphere. An aliquot of the cell suspension of macrophages (300 µL) with a cell density of 5 x 10^5 cells/mL was placed in each well. The microplate was incubated for 24 hours in the incubator with the conditions previously indicated to allow an adequate adherence and multiplication of the cells.

After that period, the cells were treated with different concentrations of extract (15 µL, 0.125 - 8 mg/mL) and incubated for one hour, with the range of concentrations tested being 6.25 - 400 µg/mL. Stimulation was performed with the addition of 30 µL of the liposaccharide solution (1 µg/mL) and incubated for an additional 24 hours. Dexamethasone (50 mM) was used as a positive control and samples in the absence of liposaccharide solution were used as a negative control. Quantification of nitric oxide was performed using Griess reagent (Promega, Winsconsin, US) and through the nitrite calibration curve. The nitric oxide produced was determined by reading absorbances at 540 nm (ELx800 Biotek microplate reader, Bio-Tek Instruments, Inc., Vermont, US) and by comparison with the standard calibration line. The results were calculated through the graphical representation of the percentage of inhibition of nitric oxide production versus the sample concentration, and expressed in relation to the concentration that causes the 50% inhibition of nitric oxide production - IC_{50} (Jabeur *et al.*, 2016).

4. Results and discussion

4.1. Experimental data for response surface method

Mathematical modeling allows the representation of different systems using an equation or a set of equations, consisting of a finite number of variables and parameters. The main goal of the models presented in this work was to study the variation of phenolic composition present in *E. globulus* leaves under different extraction conditions through a simulation that approximates the real characteristics and, thereafter, finding parameter values to achieve the optimal responses. For the model to fulfill the referred purpose, it was properly validated within its applicability domain and presented to comply with a satisfactory forecast range, consistent with the experimental data.

Table 4.1 and Table 4.2 summarize the experimental plan for the two extraction methods, including the independent variables (time, temperature/power, and solvent composition) and their ranges, as well as the response variables (total yield – Y, total phenolic acids content – TPA, total flavonoids content – TFC, and total phenolic content – TPC). Table 4.3 and Table 4.4 show the regression coefficients obtained for each model according to a second-degree polynomial function and the correlation parameters: coefficient of determination[3] (R^2) and adjusted R-squared[4] (R_A^2). The value determined for R^2 for all significant variables was greater than 0.95, which indicates that the model is considered adequate to represent the variability of the data The non-significant variables for the prediction were discarded for a more compact model, representing by *ns* (non-significant). The quality of data fitness was based on the analysis of variance – ANOVA (Table 4.5), in which the *F*-values confirm the satisfactory data fitting. Equations were set up to predict yield values and content of total phenolic compounds, phenolic acids, and flavonoids, composed of the significant regression coefficients calculated at the 95% confidence level.

[3] Coefficient of determination (R^2): in this work, it indicates the level of adequacy of regression models in relation to the experimental data, expressed in percentage of variance.

[4] Adjusted R-squared (R_A^2): adjusted version of R squared based on the number of coefficients $\hat{\beta}$ in the regression model.

Table 4.1: Coded variables, natural values, ranges, and experimental RSM results of the CCCD for the optimization of the three main variables involved (x_1, x_2, and x_3) in HAE for all the response values assessed: extraction yield, TPA, TFC, and TPC. Two replicates were performed for each combination.

Coded Values			Natural values			Y	TPA	TFC	TPC
x_1	x_2	x_3	t (min)	T (°C)	S (% EtOH)	(mg.g^{-1} dw)	(mg.g^{-1} dw)	(mg.g^{-1} dw)	(mg.g^{-1} dw)
1	-1	1	100	37	80	30.37	8.48	5.33	13.81
1	-1	-1	100	37	20	22.43	9.71	5.29	15.00
-1	-1	-1	40	37	20	22.77	9.06	5.10	14.16
-1	-1	1	40	37	80	28.40	8.08	4.50	12.57
-1.68	-1.68	-1.68	20	25	0	18.77	7.07	4.92	11.98
-1.68	-1.68	1.68	20	25	100	18.60	5.29	2.39	7.68
1.68	-1.68	1.68	120	25	100	22.33	6.89	2.72	9.61
1.68	-1.68	-1.68	120	25	0	19.67	7.73	5.13	12.86
-1.68	1.68	1.68	20	85	100	25.27	9.92	3.76	13.68
0	1.68	0	70	85	50	32.17	14.77	5.12	19.90
1.68	1.68	1.68	120	85	100	36.03	9.11	4.34	13.44
-1	1	1	40	73	80	32.17	13.36	5.05	18.41
-1	1	-1	40	73	20	24.47	13.77	5.53	19.29
1	1	1	100	73	80	35.57	13.87	5.98	19.85
1	1	-1	100	73	20	25.70	15.10	5.44	20.54
0	0	-1.68	70	55	0	20.30	11.15	5.65	16.80
0	0	1.68	70	55	100	24.87	8.31	3.60	11.92
1.68	0	0	120	55	50	29.63	16.98	7.48	24.46
-1.68	0	0	20	55	50	27.47	16.67	6.35	23.01
0	0	0	70	55	50	29.03	12.03	5.69	17.71
0	0	0	70	55	50	30.20	12.29	5.84	18.13
0	0	0	70	55	50	29.30	13.50	5.80	19.30
0	0	0	70	55	50	28.70	13.57	5.79	19.35
0	0	0	70	55	50	28.87	13.64	5.87	19.51
0	0	0	70	55	50	29.03	13.58	5.69	19.27
0	0	0	70	55	50	29.70	12.88	5.78	18.67
1.68	1.68	-1.68	120	85	0	22.13	21.12	6.18	27.30
-1.68	1.68	-1.68	20	85	0	22.37	18.33	6.32	24.64

TPA – Total Phenolic Acids, TFC – Total Flavonoids Content, TPC – Total Phenolic Content

Table 4.2: Coded variables, natural values, ranges, and experimental RSM results of the CCCD for the optimization of the three main variables involved (x_1, x_2, and x_3) in UAE for all the response values assessed: extraction yield, TPA, TFC, and TPC. Two replicates were performed for each combination.

Coded Values			Natural values			Y	TPA	TFC	TPC
x_1	x_2	x_3	t (min)	P (W)	S (% EtOH)	(mg.g^{-1} dw)	(mg.g^{-1} dw)	(mg.g^{-1} dw)	(mg.g^{-1} dw)
-1.68	-1.68	-1.68	3	100	0	11.38	7.36	1.94	9.30
1.68	-1.68	-1.68	45	100	0	15.74	9.84	4.01	13.85
0	0	-1.68	24	300	0	18.73	9.81	3.50	13.31
1.68	1.68	-1.68	45	500	0	22.16	9.61	3.78	13.39
-1.68	1.68	-1.68	3	500	0	18.81	9.64	3.00	12.63
1	-1	-1	36	180	20	19.21	10.77	3.70	14.47
-1	-1	-1	12	180	20	19.14	9.56	3.16	12.72
-1	1	-1	12	420	20	21.32	12.49	3.92	16.41
1	1	-1	36	420	20	22.30	12.07	3.67	15.74
0	1.68	0	24	500	50	27.17	9.47	3.96	13.44
1.68	0	0	45	300	50	22.74	8.98	3.91	12.89
-1.68	0	0	3	300	50	17.55	6.99	3.34	10.33
0	-1.68	0	24	100	50	15.26	9.14	3.42	12.55
1	-1	1	36	180	80	17.31	6.21	3.33	9.54
-1	-1	1	12	180	80	16.57	5.28	2.57	7.85
-1	1	1	12	420	80	22.36	7.54	3.28	10.82
1	1	1	36	420	80	25.05	8.02	3.60	11.62
-1.68	-1.68	1.68	3	100	100	8.34	0.59	0.92	1.50
1.68	-1.68	1.68	45	100	100	13.43	1.08	1.25	2.33
-1.68	1.68	1.68	3	500	100	17.29	4.28	2.15	6.43
1.68	1.68	1.68	45	500	100	23.17	3.92	2.38	6.29
0	0	1.68	24	300	100	18.76	3.81	1.59	5.40
0	0	0	24	300	50	24.60	10.09	3.64	13.73
0	0	0	24	300	50	24.42	10.27	3.63	13.90
0	0	0	24	300	50	22.46	10.07	3.62	13.68
0	0	0	24	300	50	24.36	10.06	3.61	13.68
0	0	0	24	300	50	24.48	10.15	3.61	13.75
0	0	0	24	300	50	24.78	9.99	3.68	13.66

TPA – Total Phenolic Acids, TFC – Total Flavonoids Content, TPC – Total Phenolic Content

Table 4.3: A – Fitting coefficients of the regression model for the HAE assessed according to the CCCD with 5 range levels (Table 3.1); B – Optimal conditions for HAE extraction and maximum response values obtained.

Response variable	A: Fitting coefficients obtained after applying the RSM equation										B: Optimal conditions and response values				
	Intercept	Linear effect			Quadratic effect			Interactive effect							
	b_0	b_1t	b_2T	b_3S	$b_{11}t^2$	$b_{22}T^2$	$b_{33}S^2$	$b_{12}tT$	$b_{13}tS$	$b_{23}TS$	R^2	*t* (min)	*T* (°C)	*S* (%)	Optimum
Y	29.33 ± 1.16	0.98 ± 0.35	1.88 ± 0.35	1.96 ± 0.35	ns	Ns	-2.21 ± 0.58	0.27 ± 0.18	0.61 ± 0.18	0.62 ± 0.18	0.9113	120.0	85.0	77.3	36.74 ± 2.47
TPA	12.79 ± 0.8	0.29 ± 0.24	2.44 ± 0.24	-1.31 ± 0.24	1.33 ± 0.40	-0.94 ± 0.40	-1.18 ± 0.40	ns	ns	-0.69 ± 0.12	0.9392	120.0	85.0	18.7	19.76 ± 1.17
TFC	5.77 ± 0.29	0.15 ± 0.09	0.36 ± 0.09	-0.54 ± 0.09	0.40 ± 0.14	-0.45 ± 0.14	-0.41 ± 0.14	ns	0.06 ± 0.04	ns	0.9009	120.0	62.1	33.6	7.34 ± 0.15
TPC	18.56 ± 0.96	0.44 ± 0.29	2.79 ± 0.29	-1.85 ± 0.29	1.73 ± 0.48	-1.39 ± 0.48	-1.59 ± 0.48	ns	ns	-0.66 ± 0.15	0.9421	120.0	76.5	25.0	26.62 ± 1.68

ns – non-significant coefficient; R2 – correlation coefficient. Optimum values are expressed in $mg.g^{-1}$ dw

Table 4.4: A – Fitting coefficients of the regression model for the UAE assessed according to the CCCD with 5 range levels (Table 3.1); B – Optimal conditions for UAE extraction and maximum response values obtained.

Response variable	A: Fitting coefficients obtained after applying the RSM equation										B: Optimal conditions and response values				
	Intercept	Linear effect			Quadratic effect			Interactive effect							
	b_0	b_1t	b_2P	b_3S	$b_{11}t^2$	$b_{22}P^2$	$b_{33}S^2$	$b_{12}tP$	$b_{13}tS$	$b_{23}PS$	R^2	*t* (min)	*P* (W)	*S* (%)	Optimum
Y	23.34 ± 0.90	1.23 ± 0.27	2.58 ± 0.27	2.57 ± 0.27	-0.83 ± 0.45	-0.45 ± 0.45	-1.33 ± 0.45	ns	0.16 ± 0.14	0.31 ± 0.14	0.9411	33.0	500.0	54.0	26.88 ± 1.48
TPA	10.07 ± 0.48	0.27 ± 0.15	0.64 ± 0.15	-2.00 ± 0.15	-0.52 ± 0.24	Ns	-0.94 ± 0.24	-0.16 ± 0.07	-0.08 ± 0.07	0.17 ± 0.07	0.9632	25.0	500.0	22.6	11.93 ± 0.42
TFC	3.73 ± 0.18	0.22 ± 0.05	0.22 ± 0.05	-0.41 ± 0.05	ns	Ns	-0.41 ± 0.09	-0.07 ± 0.03	-0.08 ± 0.03	ns	0.9333	45.0	500.0	34.0	4.39 ± 0.06
TPC	13.80 ± 0.54	0.49 ± 0.16	0.86 ± 0.16	-2.42 ± 0.16	-0.55 ± 0.27	Ns	-1.35 ± 0.27	-0.23 ± 0.08	ns	0.24 ± 0.08	0.9705	26.45	500.0	27.2	16.03 ± 0.53

ns – non-significant coefficient; R2 – correlation coefficient. Optimum values are expressed in $mg.g^{-1}$ dw.

Table 4.5: ANOVA for heat-assisted and ultrasound-assisted extractions.

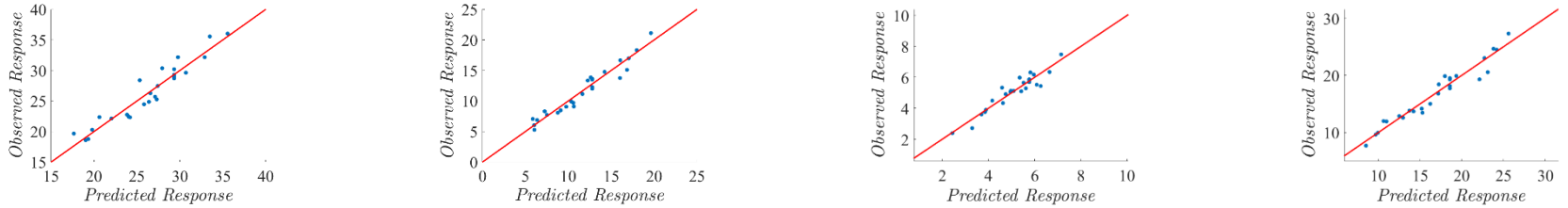
Heat-assisted Extraction					
		SS	DF	MS	*F*-value
Y	Model	557.50	9	61.94	20.55
	Residual	54.26	18	3.01	
	Total	611.76	27		
TPA	Model	396.15	9	44.02	30.89
	Residual	25.65	18	1.42	
	Total	421.80	27		
TFC	Model	30.15	9	3.35	18.17
	Residual	3.32	18	0.18	
	Total	33.46	27		
TPC	Model	604.25	9	67.14	32.52
	Residual	37.16	18	2.06	
	Total	641.41	27		
Ultrasound-assisted Extraction					
		SS	DF	MS	*F*-value
Y	Model	557.50	9	61.94	20.55
	Residual	54.26	18	3.01	
	Total	611.76	27		
TPA	Model	396.15	9	44.02	30.89
	Residual	25.65	18	1.42	
	Total	421.80	27		
TFC	Model	30.15	9	3.35	18.17
	Residual	3.32	18	0.18	
	Total	33.46	27		
TPC	Model	604.25	9	67.14	32.52
	Residual	37.16	18	2.06	
	Total	641.41	27		

SS – Sum of Squares, DF – Degrees of Freedom, MS – Mean of Squares, Y – Total Yield, TPA – Total Phenolic Acids, TFC – Total Flavonoids, TPC – Total Phenolic Content.

Residual analysis and normality analysis (by the Kolmogorov-Smirnov test) for each optimized response are graphically shown in Figure 4.1 and Figure 4.2. These tests verified the suitability of each model for quantitative predictions by the satisfactory agreement between predicted and measured values. The results also showed that the choice of independent variables (time, temperature or power, and solvent) and the setting of constant parameters such as solid-liquid ratio and particle size of powdered leaves were adequate for the extraction of *E. globulus*.

TOTAL YIELD (Y)	PHENOLIC ACIDS CONTENT (TPA)	FLAVONOIDS CONTENT (TFC)	TOTAL PHENOLIC CONTENT (TPC)

A: Statistical distribution (Kolmogorov-Smirnov)



B: Residual plots

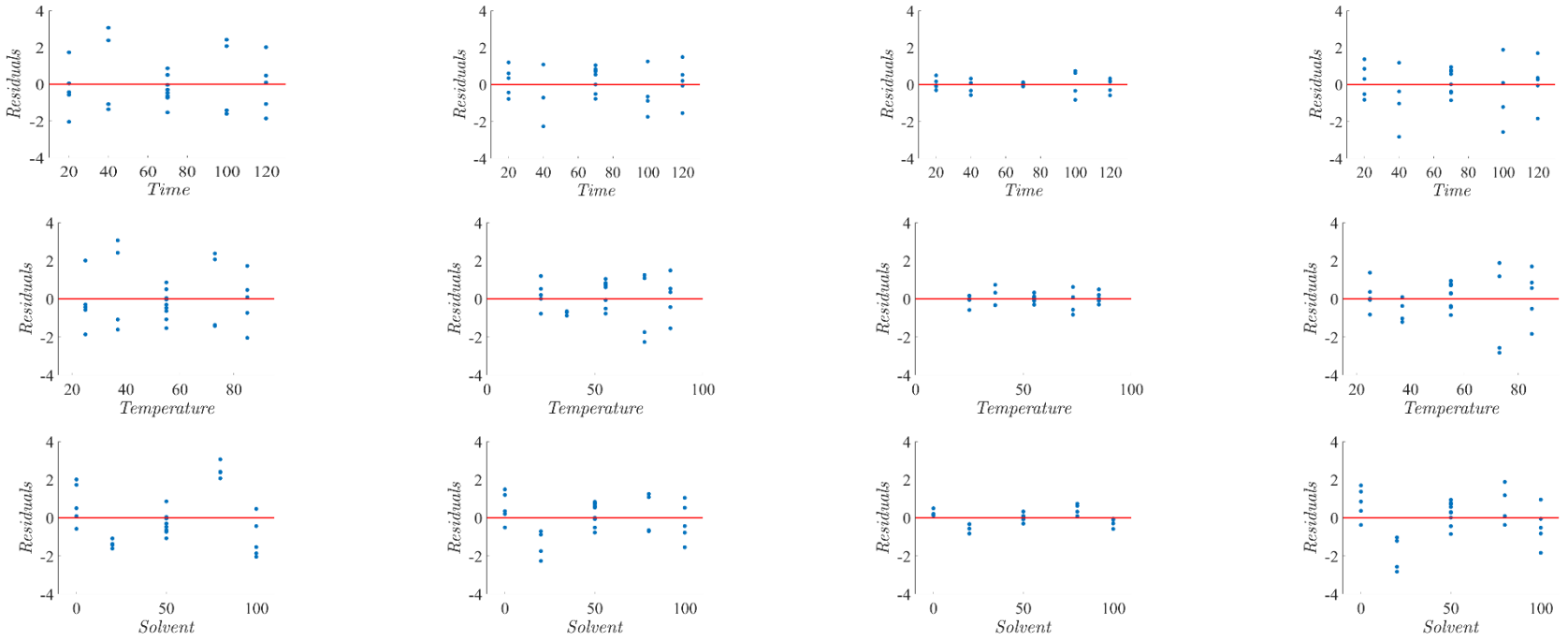
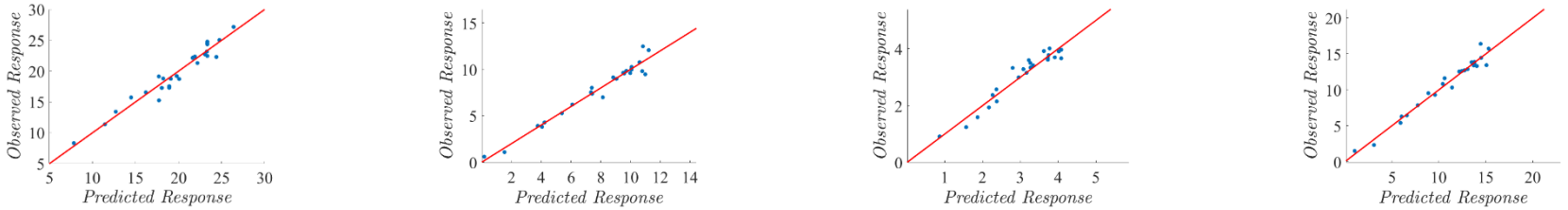


Figure 4.1: A – Statistical distribution of the results obtained in comparison with the responses calculated by the regression model; B – Residuals for each independent variable analyzed in the HAE extraction.

TOTAL YIELD (Y)	PHENOLIC ACIDS CONTENT (TPA)	FLAVONOIDS CONTENT (TFC)	TOTAL PHENOLIC CONTENT (TPC)

A: Statistical distribution (Kolmogorov-Smirnov)



B: Residual plots

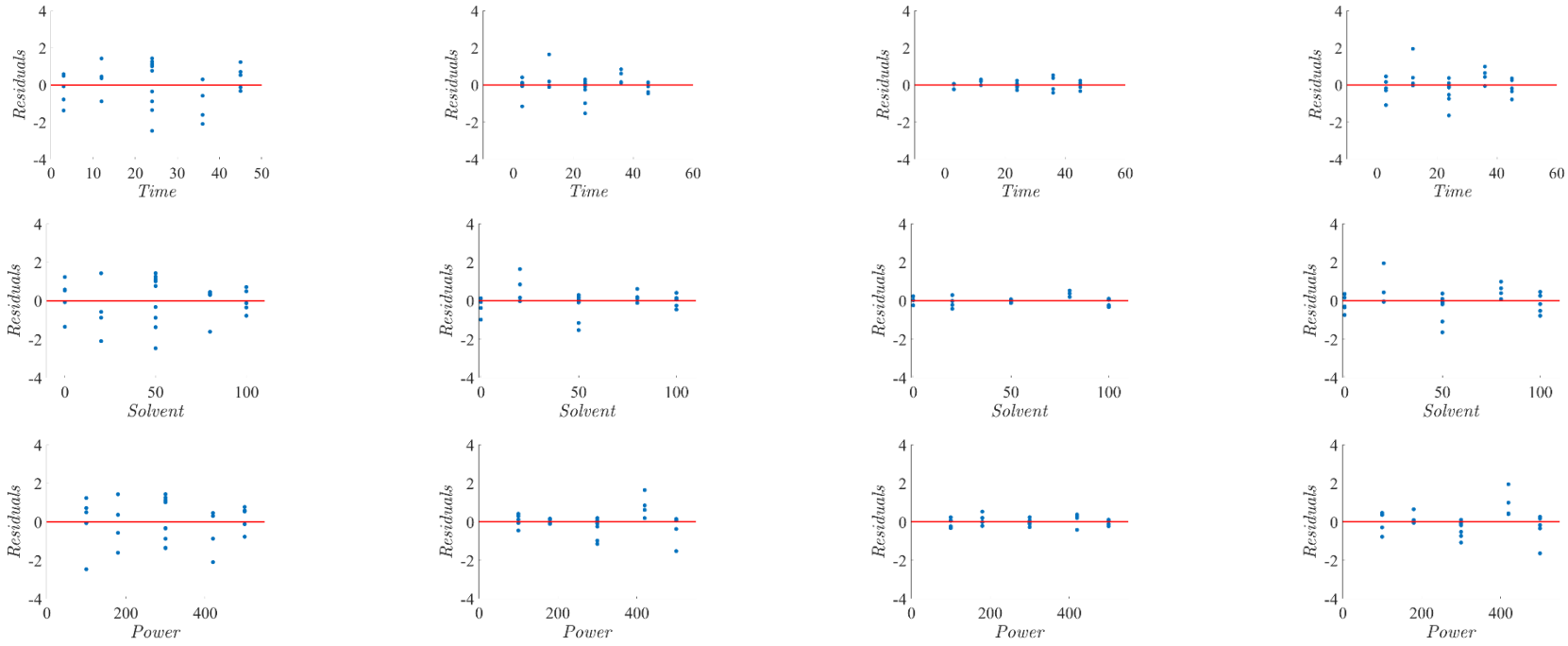


Figure 4.2: A – Statistical distribution of the results obtained in comparison with the responses calculated by the regression model; B – Residuals for each independent variable analyzed in the HAE extraction

4.1.1. Dynamic Response Surface Methodology

The optimization results with Dynamic RSM were evaluated in comparison to those previously reported using the traditional RSM. The cluster analysis for each response variable was performed considering the means and medoids for the outputs (optimal responses). The representation for k-clustering methods is illustrated in Figure 4.3. The bootstrap analysis displayed in Figures 4.4-9 makes inferences about the results, graphically represented in terms of means (*k-means*) and medoids (*k-medoids*) considering the optimal responses achieved using the method.

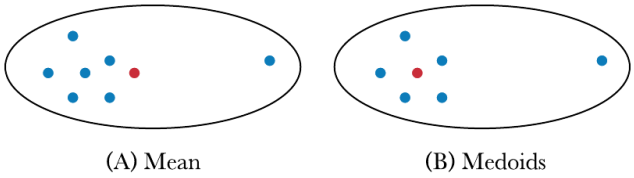


Figure 4.3: Mean and Medoid in 2D space representation. In both figures, the data are represented by blue dots, being the rightmost point, an outlier and the red point represents the centroid point found by *k-mean* or *k-medoid* methods. Adapted from Jin and Han (2017).

The histograms for the RSM Dynamic response set and the bootstrap distribution of the mean (1000 re-samplings) in Figure 4.10 and Figure 4.11 display the frequency of the different optimal combinations estimated by the method. The combinations shown on the horizontal axis of each histogram correspond to the mean of the corresponding class (bar). For all studied responses, the identified optimal point belongs to the highest frequency range for the output data. On the other hand, the values of the optimal conditions in the bootstrap graphs have narrow amplitudes, thus obtaining histograms with similar classes. The box plots of the Dynamic RSM optimal response group (Figure 4.12 and Figure 4.13) show that there is a large dispersion in the optimal results obtained in the 100 runs, for all evaluated responses (Y, TPA, TFC, and TPC). To find the optimal point, 15 to 18 combinations were needed, in contrast to the traditional RSM which uses 28 combinations.

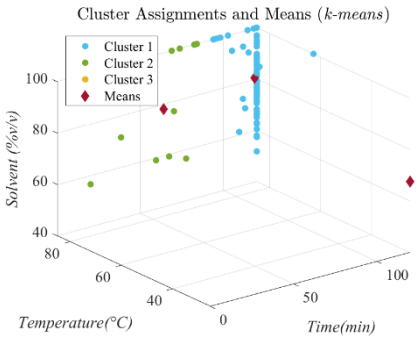
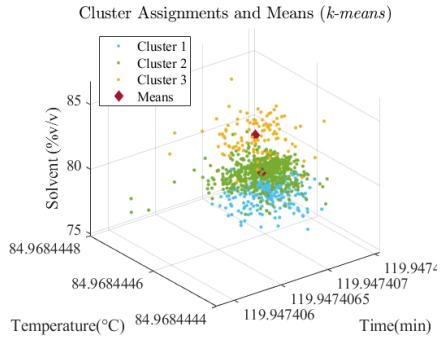
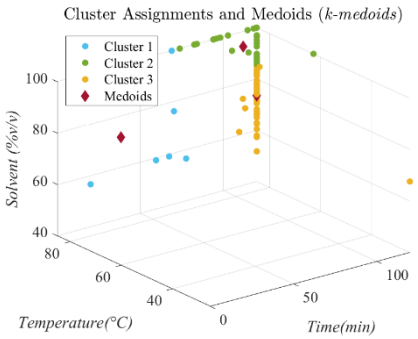
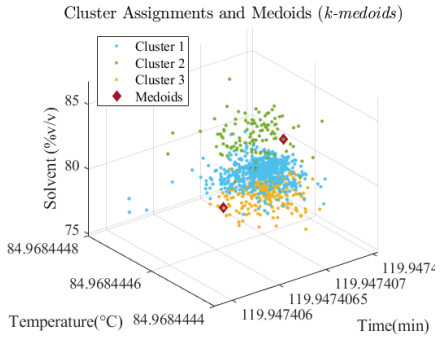
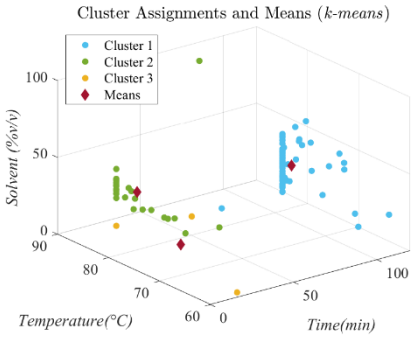
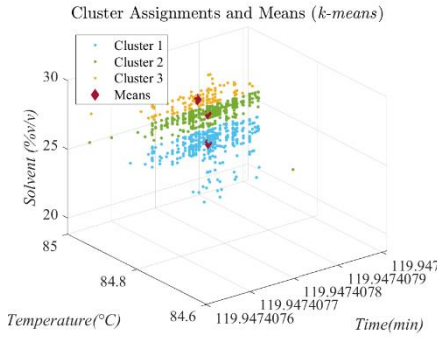
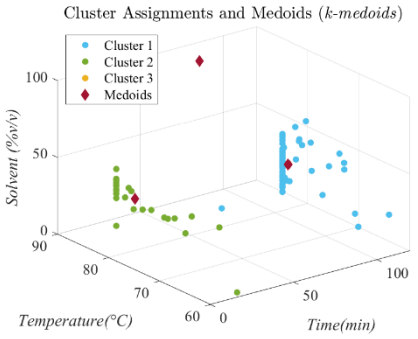
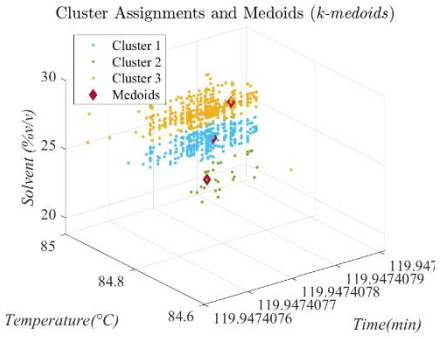
	k-means		*k-medoids*	
	Output	Bootstrap	Output	Bootstrap
Y				
TPA				

Figure 4.4: *K-means* and *k-medoids* clustering plots of heat-assisted extraction (HAE) for Yield and Total Phenolic Acids Content (TPA).

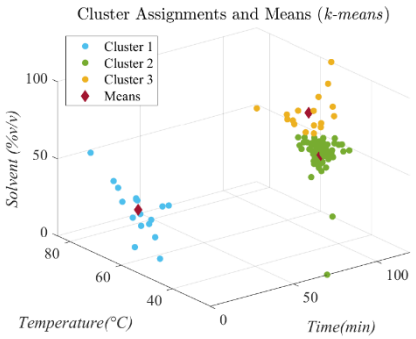
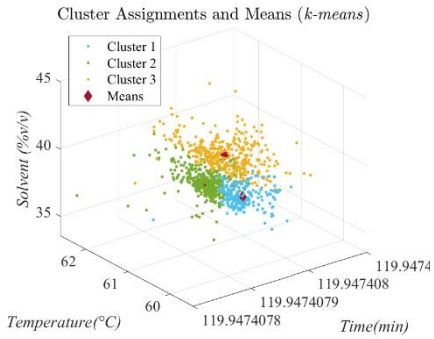
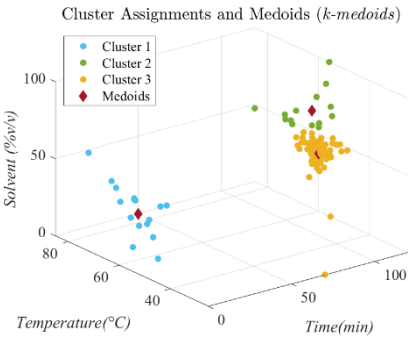
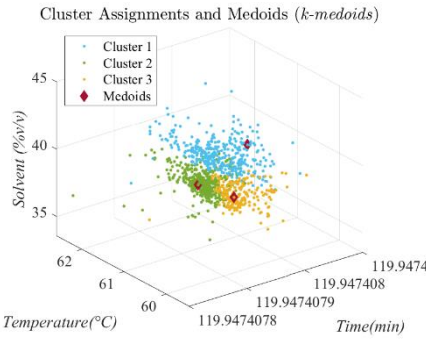
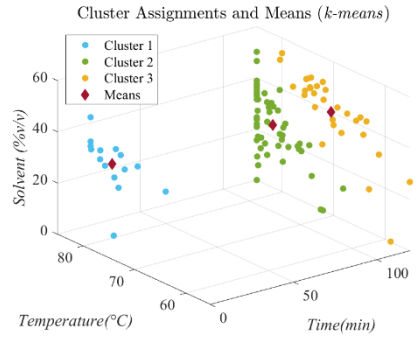
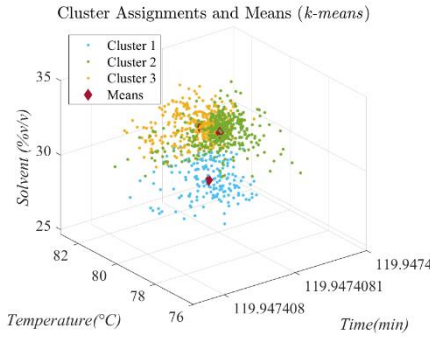
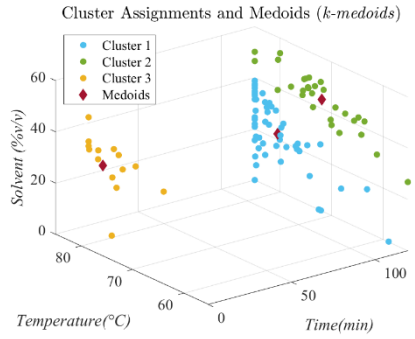
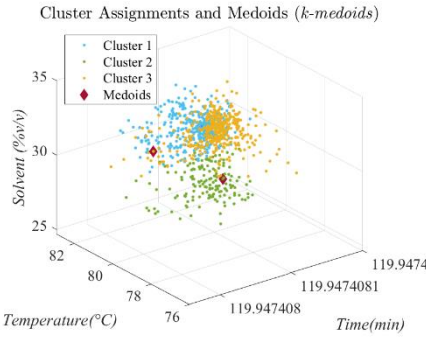
	k-means		*k-medoids*	
	Output	Bootstrap	Output	Bootstrap
TFC				
TPC				

Figure 4.5: *K-means* and *k-medoids* clustering plots of heat-assisted extraction (HAE) for Total Flavonoids Content (TFC) and Total Phenolic Content (TPC).

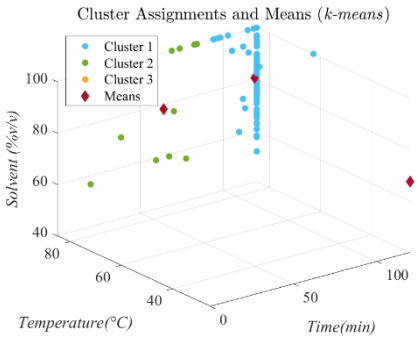
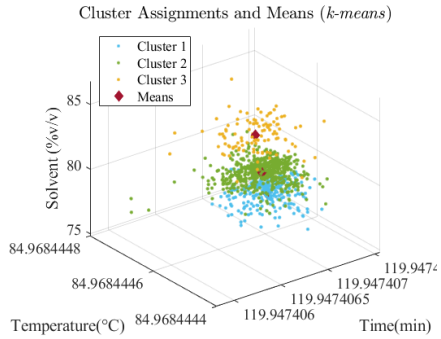
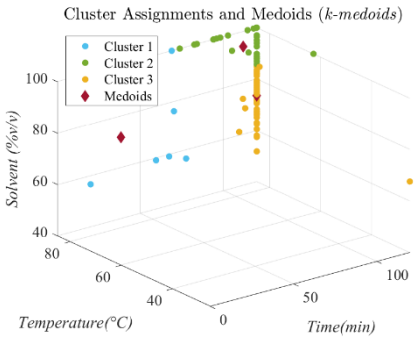
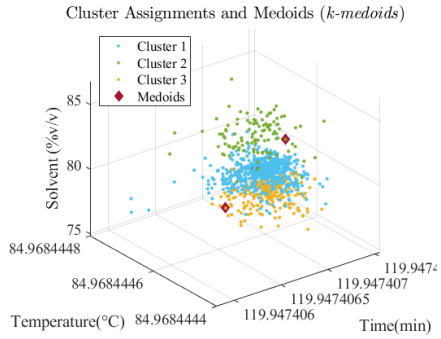
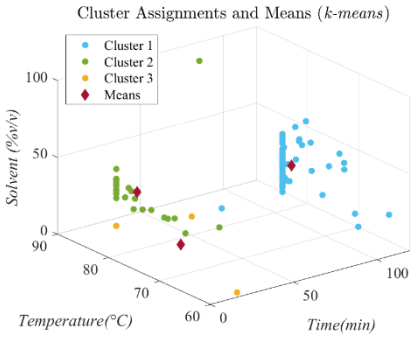
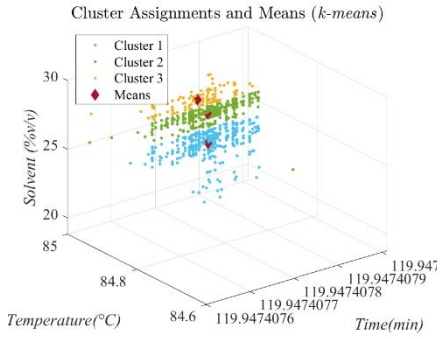
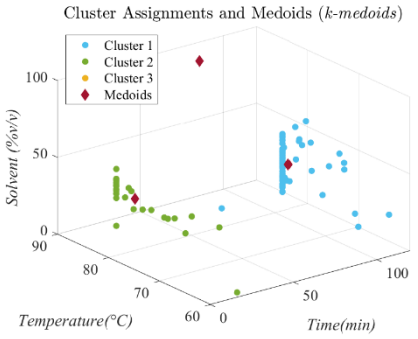
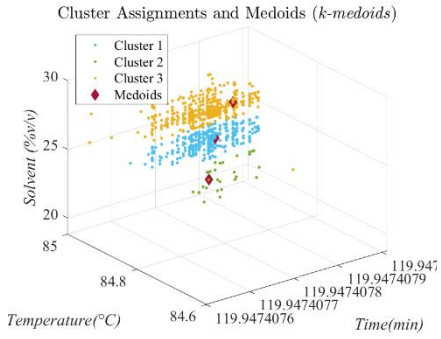
	k-means		*k-medoids*	
	Output	Bootstrap	Output	Bootstrap
Y				
TPA				

Figure 4.6: *K-means* and *k-medoids* clustering plots of heat-assisted extraction (HAE) for Yield and Total Phenolic Acids Content (TPA).

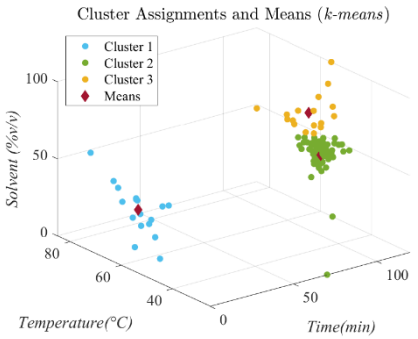
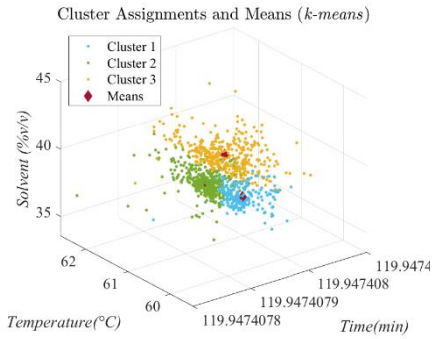
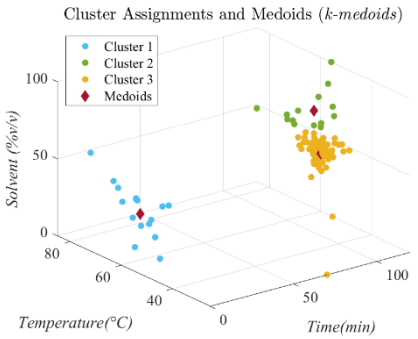
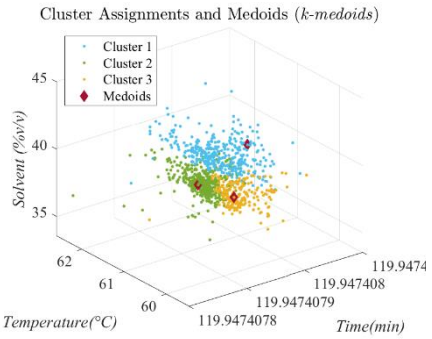
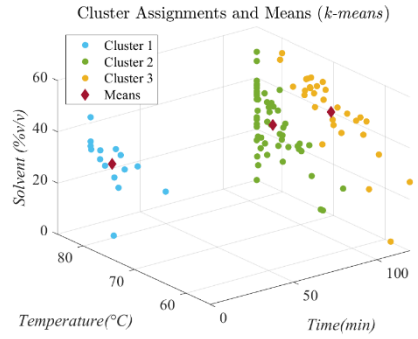
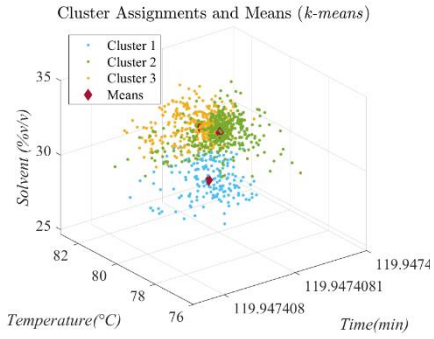
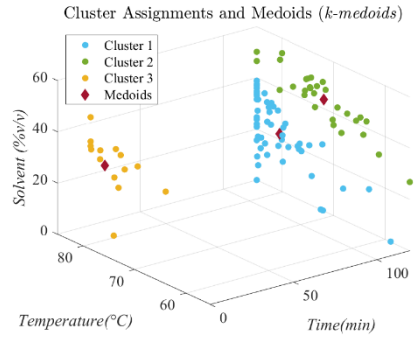
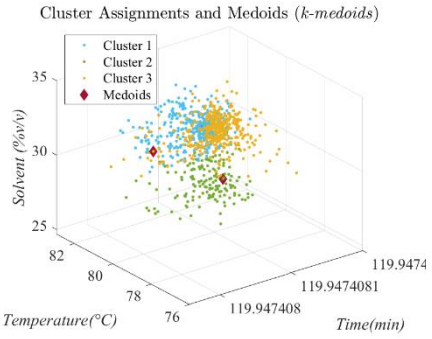
	k-means		*k-medoids*	
	Output	Bootstrap	Output	Bootstrap
TFC				
TPC				

Figure 4.7: *K-means* and *k-medoids* clustering plots of heat-assisted extraction (HAE) for Total Flavonoids Content (TFC) and Total Phenolic Content (TPC).

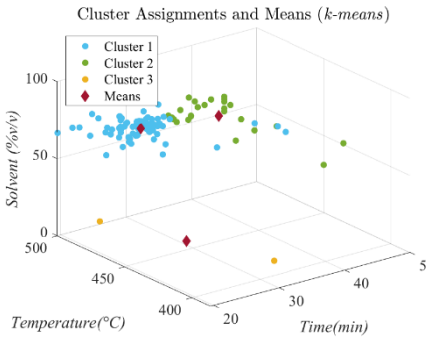
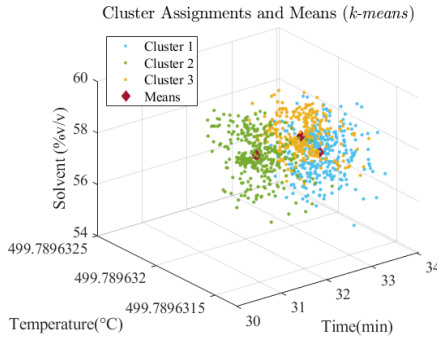
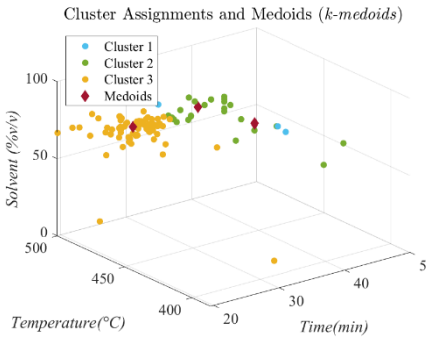
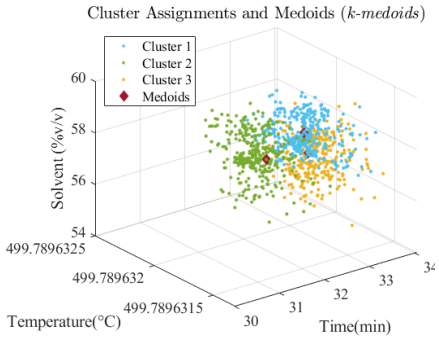
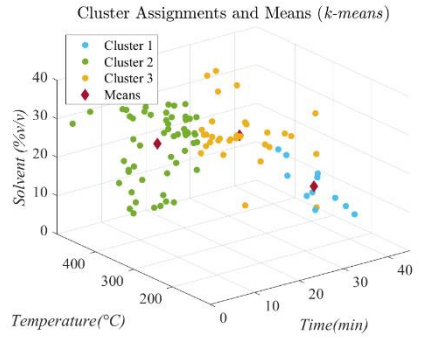
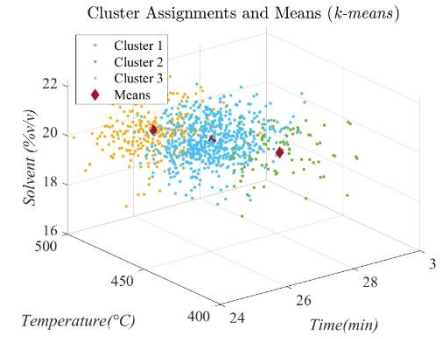
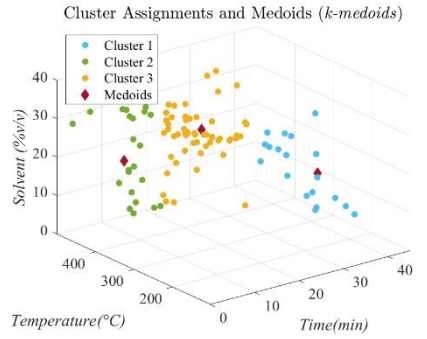
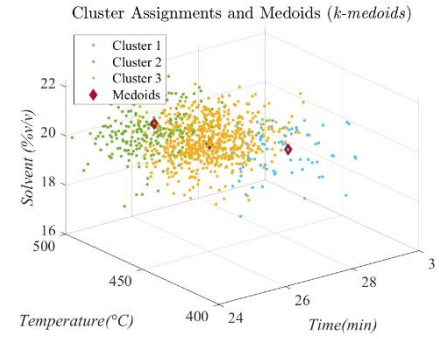
	k-means		*k-medoids*	
	Output	Bootstrap	Output	Bootstrap
Y				
TPA				

Figure 4.8: *K-means* and *k-medoids* clustering plots of ultrasound-assisted extraction (UAE) for Yield and Total Phenolic Acids Content (TPA).

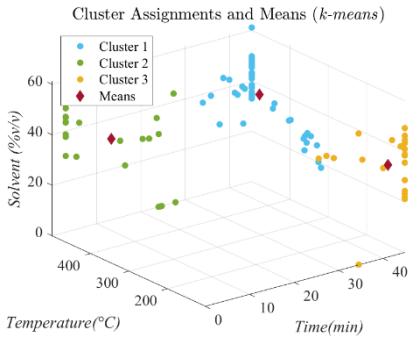
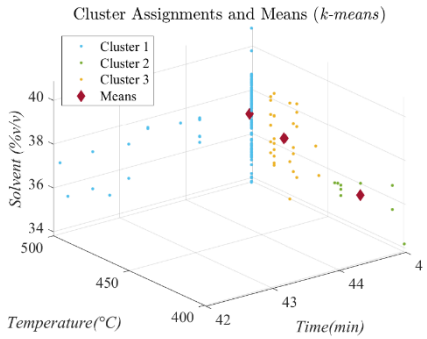
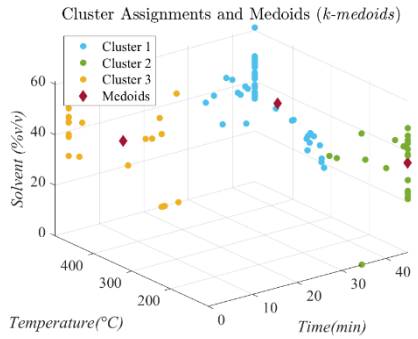
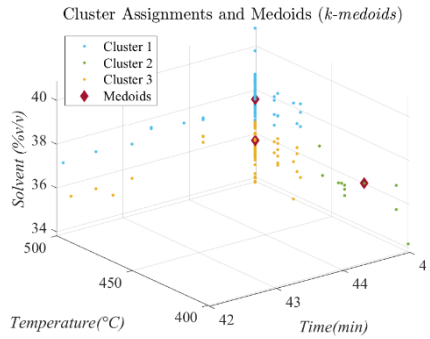
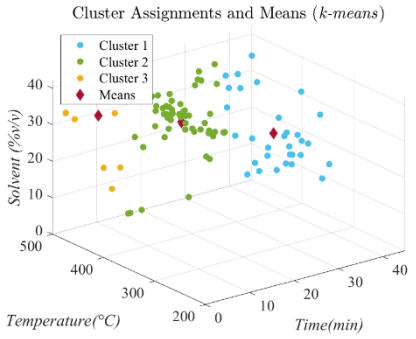
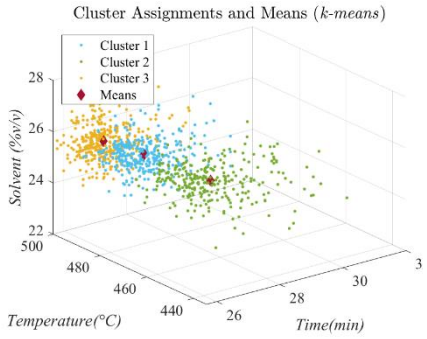
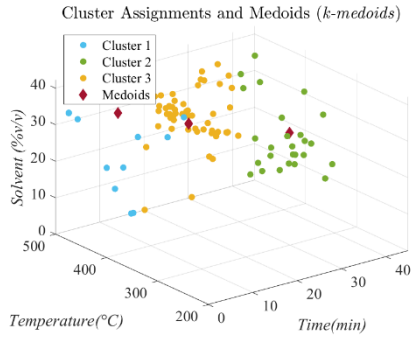
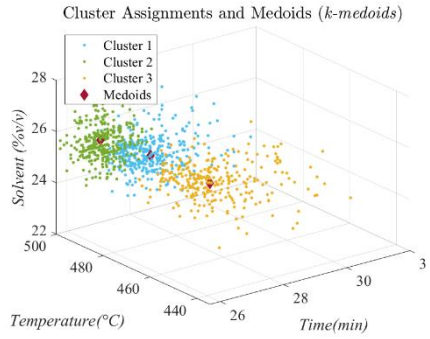
	k-means		*k-medoids*	
	Output	Bootstrap	Output	Bootstrap
TFC				
TPC				

Figure 4.9: *K-means* and *k-medoids* clustering plots of ultrasound-assisted extraction (UAE) for Total Flavonoids Content (TFC) and Total Phenolic Content (TPC).

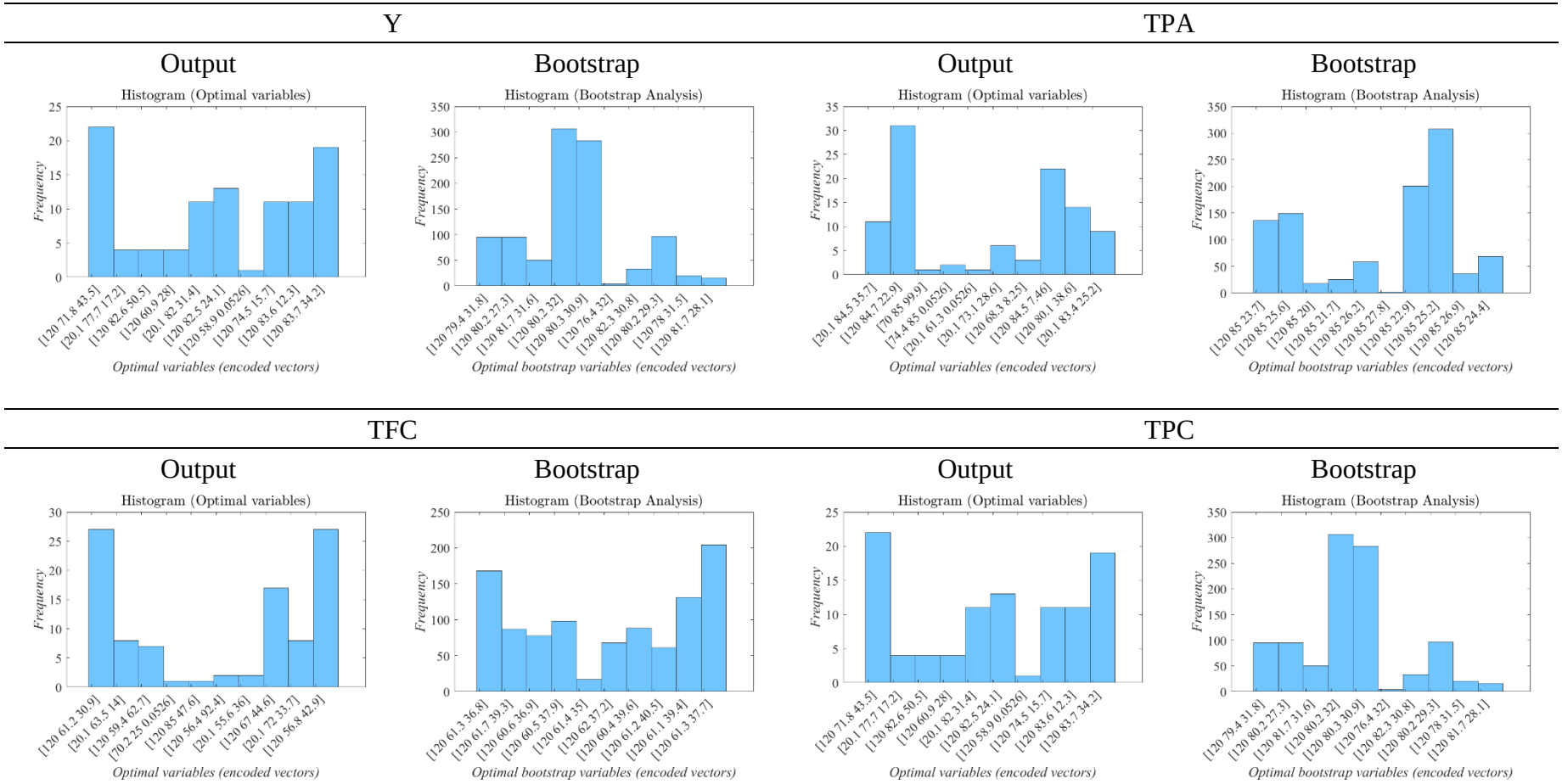


Figure 4.10: Histograms of the optimal combinations of the independent variables (time, temperature, and solvent) for heat-assisted extraction. Y – Total Yield, TPA – Total Phenolic Acids, TFC – Total Flavonoids, TPC – Total Phenolic Content.

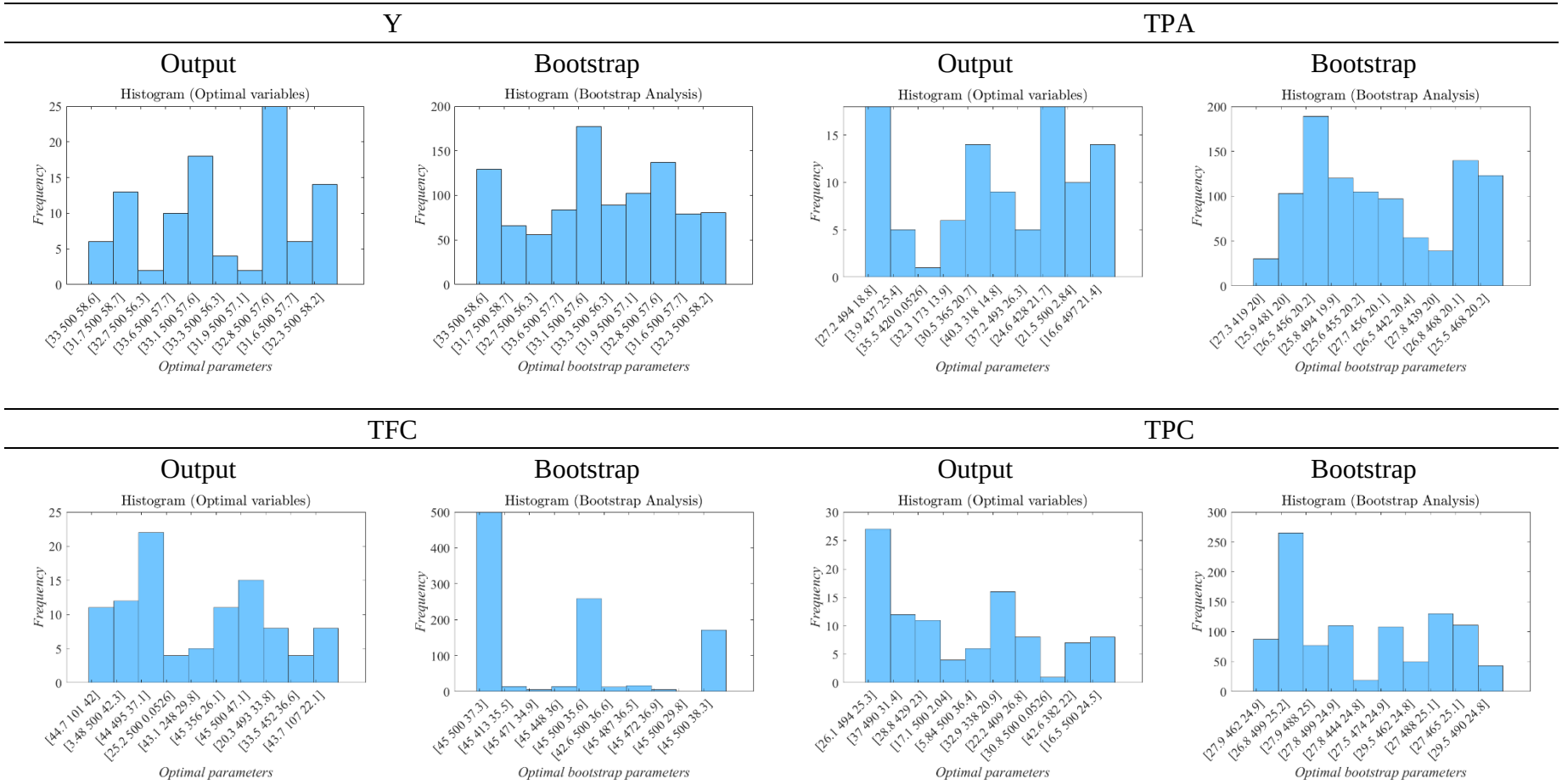


Figure 4.11: Histograms of the optimal combinations of the independent variables (time, power, and solvent) for ultrasound-assisted extraction. Y – Total Yield, TPA – Total Phenolic Acids, TFC – Total Flavonoids, TPC – Total Phenolic Content.

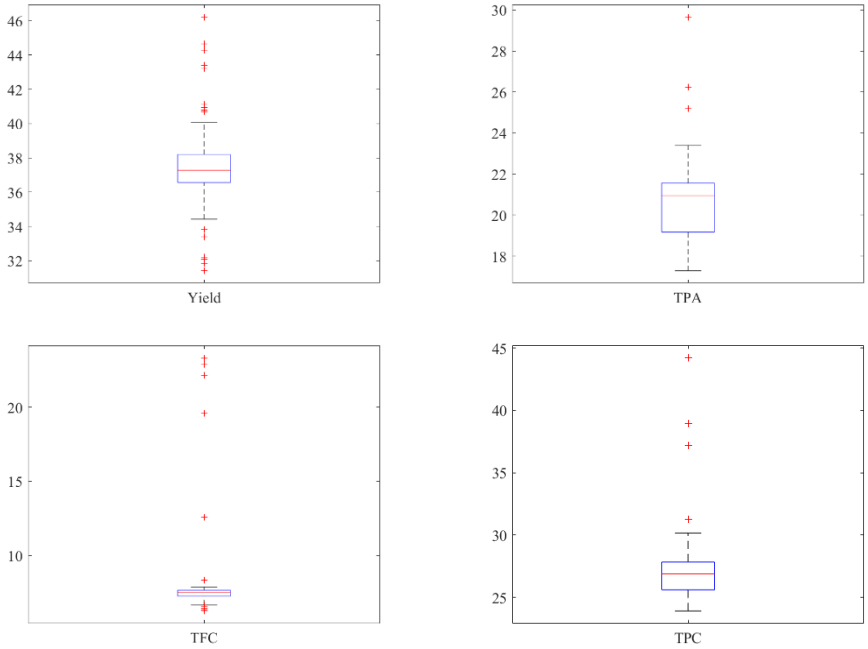


Figure 4.12: Box plot of the results for heat-assisted extraction.

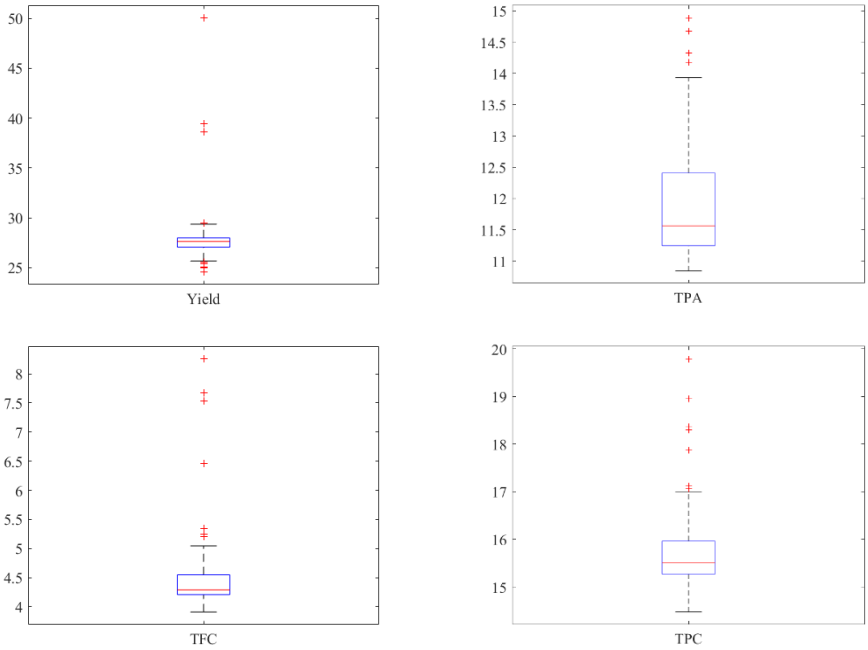


Figure 4.13: Box plot of the results for ultrasound-assisted extraction.

4.1.2. Statistical verification of the predictive models

As previously stated, the suitability of each model for quantitative predictions was verified using analysis of variance (ANOVA) for traditional RSM and bootstrap analysis for dynamic RSM. However, the agreement between predicted and actual values must be confirmed through experimental tests. Due to time constraints, this step was not performed, which is therefore one of the limitations of this work. On the other hand, the referred statistical tests proved that the choice of independent variables (time, temperature or power, and solvent) and the establishment of constant parameters such as the solid-liquid proportion and particle size of the powdered leaves were adequate for the extraction of *E. globulus* leaves.

Table 4.6: Predicted and experimental values under optimal conditions for the response variables approached (extraction yield, TPA, TFC, and TPC).

Extraction method		Response variable			
		Y (mg/g dry leaves)	TPA (mg/g dry leaves)	TFC (mg/g dry leaves)	TPC (mg/g dry leaves)
HAE	Traditional RSM	36.72 ± 2.47	19.76 ± 1.17	7.34 ± 0.15	26.62 ± 1.68
	Dynamic RSM	37.27 ± 0.93	20.94 ± 1.77	7.65 ± 0.39	26.88 ± 1.28
	Experimental value	37.13	-	-	-
UAE	Traditional RSM	26.88 ± 1.48	11.93 ± 0.42	4.39 ± 0.06	16.03 ± 0.53
	Dynamic RSM	27.63 ± 0.57	11.59 ± 0.82	4.29 ± 0.28	15.51 ± 0.45
	Experimental value	28.1	-	-	-

Y – Total Yield, TPA – Total Phenolic Acids, TFC – Total Flavonoids, TPC – Total Phenolic Content.

Therefore, the regression models proved to be accurate and reliable to predict the yield and the phenolic content (phenolic acids, flavonoids, and total) and the antioxidant capacities of ethanolic eucalyptus extract. The performance of the dynamic method was satisfactory; however, it is important to point out that it is a new approach and upgrades should be done to improve its robustness.

4.2. Response surface analysis

4.2.1. Total yield

The extraction yields presented values between 18.60 and 36.03 mg/g of dry leaves for HAE and between 8.34 and 27.17 mg/g of dry leaves for UAE (Table 4.1 and Table 4.2). The yield obtained is in agreement with the results reported in previously published studies (González-Burgos *et al.*, 2018; Gullón *et al.*, 2017) considering the variables and the conditions used in these works.

For heat-assisted extraction, the individual regression coefficients (b_0, b_1 and b_3) significantly influenced the extraction yield, among them, b_3, corresponding to solvent proportion, is the most significant. The interaction time-solvent and temperature-solvent showed great influence among the interactive factors. Regarding the quadratic factors, only the solvent coefficient influences the yield.

The level of influence of the factors is displayed in Figure 4.14, in which the increase in temperature and the time of contact of the matrix with the solvent implies an increase in total yield. However, the yield is highly influenced by the proportion of ethanol in the solvent, since, for temperature and maximum extraction time (85 °C and 120 min), 76.8% of ethanol in the solvent was predicted to achieve an optimal yield: 36.72 mg extract/g of leaves.

The result is consistent with data reported by Gullón *et al.* (2017), which showed that the extraction process is favored by high temperatures, long contact time, and ethanol concentration, since the solubility of the compounds, the diffusivity of the solvent, and the weakening of the tissue are improved, allowing a better permeability of the active fraction present in *E. globulus* leaves.

Ultrasound-assisted extraction showed a higher yield compared to the previous technique, considering the time and proportion of ethanol, being these two factors important for cost reduction and sustainability of the process. The linear model coefficients (b_1 and b_2, correspondent to time and power) are the most influential effects for the model, being the power the most significant. The high yield can be explained by the cavitation phenomena caused by the incidence of ultrasound in the extractive medium, which increases the mass

transfer during the UAE, allowing greater penetration of the solvent in the sample matrix and, therefore, facilitating the release of extractable compounds.

The maximum yield found, considering the studied ranges, was 26.9 mg/g of dry leaves, under the following conditions: 33 min, 500 W, and 53.9% ethanol. It can be seen from the response surface obtained (Figure 4.15) that the yield shows an increasing trend over time, which may be an indication that higher yields can be obtained for longer extractions. However, subjecting leaf samples of *E. globulus* to long periods of cavitation may lead to reduced extraction performance in the studied indicators because of the degradation of phenolic compounds (Tiwari, 2015).

In general, the most important parameters during UAE are ultrasonic power and frequency, extraction time, and solvent properties. Tiwari (2015) supports that temperature is a factor that must be considered during ultrasound-assisted extraction. In this work, the UAE was performed considering a constant temperature (20 °C) given equipment limitations. However, it is an interesting approach to be considered in future works.

An additional point to be considered is the presence of a significant amount of epicuticular wax present in the outermost layer of *E. globulus* leaves. For this plant matrix, preliminary wax removal procedures can be implemented, turning it easier to obtain compounds with high added value.

4.2.2. Total phenolic acids content (TPA)

The content of phenolic acids in extracts from HAE is significantly affected by the temperature and solvent applied, as evidenced by the magnitude of the corresponding linear coefficients (b_1 and b_2). However, the concentration of ethanol (affecting the polarity of the solvent) was more crucial for the extraction yield than the temperature since the application of ethanol in high concentrations resulted in a considerable decrease in the content of these compounds. The results of the study demonstrated that the phenolic profile of the extracts (composition and content) varied significantly depending on the solvent applied. In the UAE, power and time were significant, and the solvent acts with an antagonistic effect for ethanol concentrations above 40% (Figure 4.15).

The results indicated that the content of phenolic acids depends not only on the factors mentioned above but also on the stability of phenolic acids concerning these parameters. The

TPA proved to be stable during HAE, however, the derivatives of caffeic acid present in the extract significantly degraded when subjected to extraction with ethanol with a concentration greater than 80% in both extraction methods. In addition, the slight reduction in TPA as a function of the time spent in the UAE can be explained by the degradation of some phenolic acids sensitive to sonochemical effects, such as derivatives of caffeic acid present in the extracts (Qiao *et al.*, 2013).

4.2.3. Total flavonoids content (TFC)

The TFC was positively affected by temperature and solvent for HAE, and power and solvent for UAE. Among the significant terms concerning HAE, ethanol concentration had the strongest effect on the extraction of flavonoids. Analogous to TPA, TFC increased when the ethanol concentration and temperature increased, but this increase occurred only up to the achievement of the optimal conditions. Increments in these two parameters above optimal parameter values caused a decline in TFC that corroborated the negative quadratic effect of b_{22} and b_{33} coefficients (Table 4.3). The highest TFC predicted by the model was 7.34 mg/g of dry leaves, obtained at 33.6% EtOH, 62.1 °C, and 120 min.

For ultrasound-assisted extraction, the TFC obtained represents more than half of the content from HAE (4.39 mg/g of dry leaves in this case), achieved using maximum power and time and approximately the same solvent (34% of ethanol). The positive results achieved by the UAE can be explained by the increased solubility of the cell content due to the greater penetration of the solvent into cell material through the incidence of ultrasound. Similar aspects were also observed for the extraction of flavonoids from natural sources (Bhuyan *et al.*, 2017; Gullón *et al.*, 2019). However, the effects of sonication for long periods may result in the degradation of flavonoids present in the extract, justifying the short time of extraction in comparison to HAE (Tiwari, 2015).

4.2.4. Total phenolic content (TPC)

For the heat-assisted extraction, the temperature, and the concentration of ethanol in the solvent significantly affect the phenolic content, confirmed by the magnitude of the individual coefficients and by the interaction between temperature and solvent (Table 4.3). This finding can be confirmed by the response surface graphs for TPC in (Figure 4.14). From the analysis of the graphs, it can be assumed that the TPC is positively influenced by the

increase in temperature. On the other hand, the ethanol concentration acted positively until the optimum concentration is reached, from then on, the PC content is decreasing. The solvent's binary effect on the extraction can be explained by the degree of polarity depending on its composition since the solvent has two components with different polarities (polar water and non-polar ethanol).

Concerning ultrasound-assisted extractions, the results show a synergism between power and solvent, highlighted by the positive coefficient b_{23}, despite the duality presented by the solvent effect. The optimal extraction time in this case is directly connected with the power level used. Given the wax barrier present in the leaves, the cavitation process is important for obtaining phenolic compounds, which justifies the high intensity of the ultrasounds in a shorter period. This fact, along with the values of the regression coefficients, indicate that there is a decrease in the content of total phenolic compounds when the maximum power of the equipment is used for longer periods.

In general, in both extractions, ethanol-water solvent seems to be suitable for the extraction due to the difference in the polarity of the constituents, being possible to reach a wide range of compounds of different polarities. According to Rajha *et al.* (2014), the use of the ethanol-water mixture has a synergistic effect in the extraction of phenolic compounds, that is, it is more effective than using water or ethanol alone. The fact that the TPC decreases when higher concentrations of ethanol are applied suggests that the PCs present in the extract have polar nature.

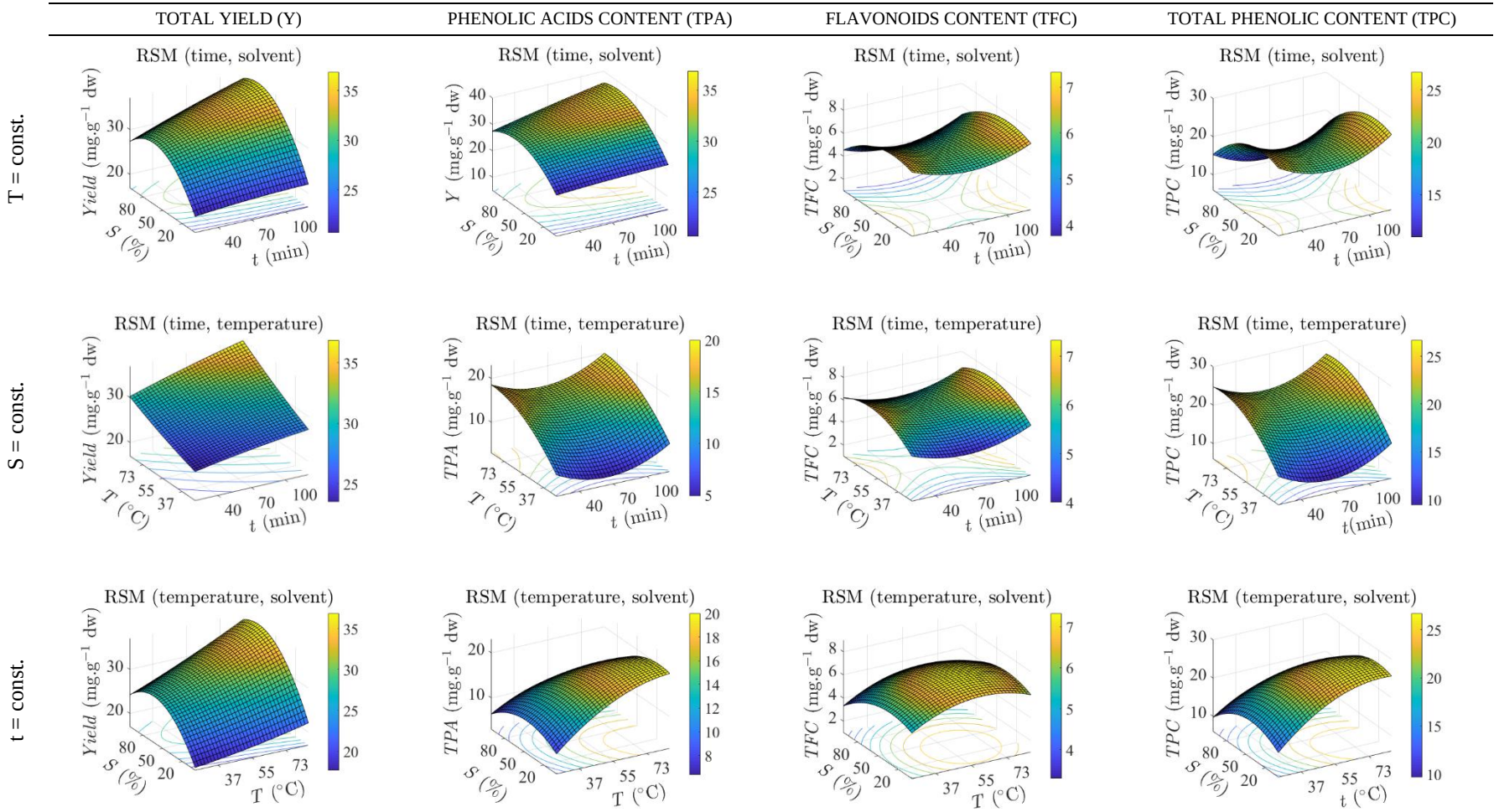


Figure 4.14:Response surface graphs as a function of each independent variable for extraction yield responses and phenolic compounds content for HAE: phenolic acids (TPA), flavonoids (TFC), and total phenolics (TPC) grouped. For representation purposes, the constant variable was positioned at the optimum of its experimental domain.

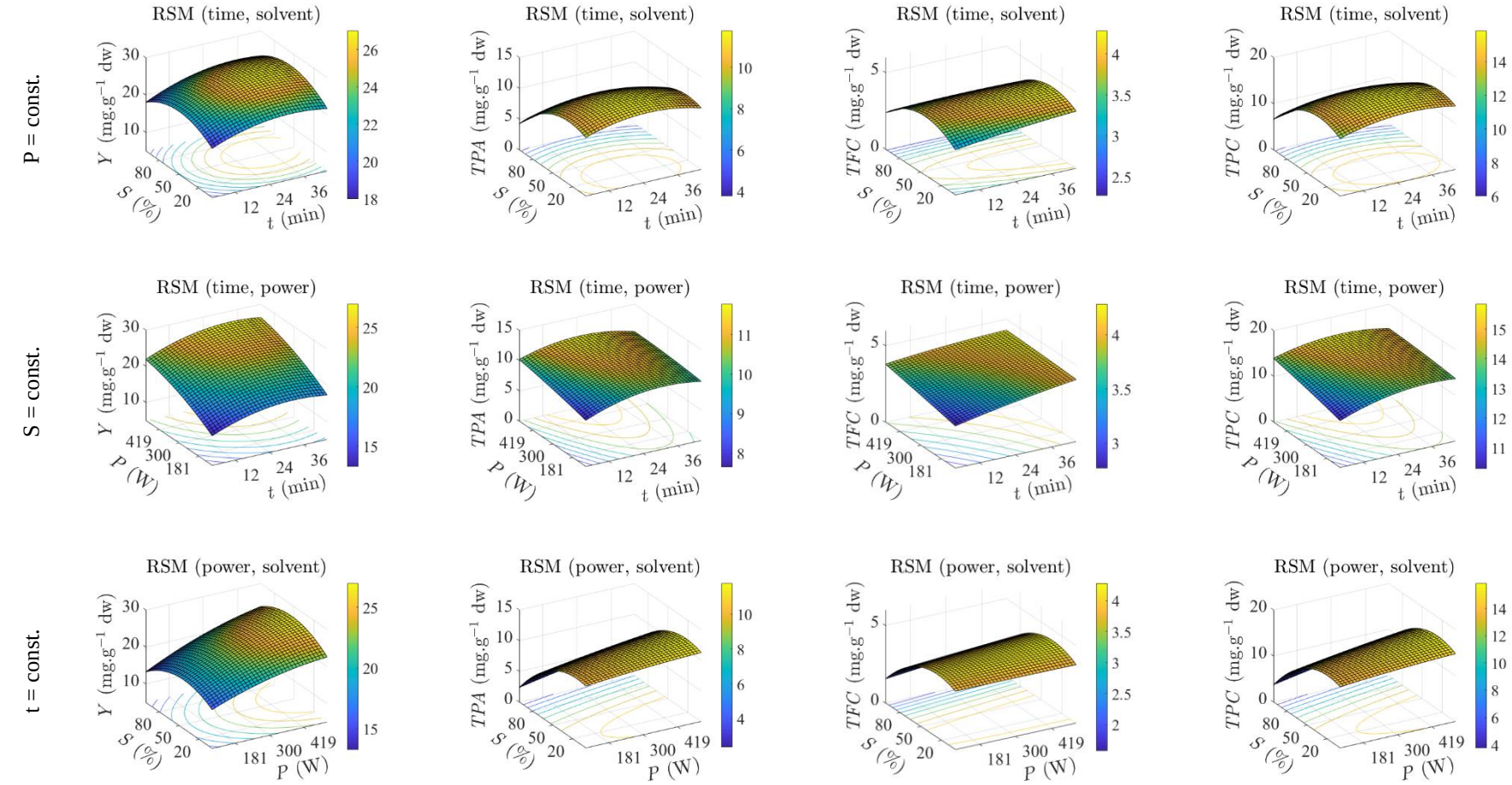


Figure 4.15:Response surface graphs as a function of each independent variable for extraction yield responses and phenolic compounds content for UAE: phenolic acids (TPA), flavonoids (TFC), and total phenolics (TPC) grouped. For representation purposes, the constant variable was positioned at the optimum of its experimental domain.

4.3. Characterization of phenolic content

The main phenolic compounds were identified by tentative considering their UV-*vis* spectra, by high-performance liquid chromatography coupled to photodiode array detector and electrospray ionization mass spectrometer (HPLC-DAD-ESI/MS) analysis. Each compound was evaluated from peak areas in the chromatographic profile, using external calibration curves established with *p*-hydroxybenzoic and caffeic acid (at 280 nm) for phenolic acids and their derivatives, terphenyl derivatives, and gallotannins, and with quercetin 3-*O*-glucoside (at 370 nm) for flavonoids, thus two fractions of compounds (F1 and F2) were distinguished based on the wavelength applied. Based on the sum of the quantifications performed for each fraction, it was possible to determine the content of total phenolic compounds (TPC), phenolic acids (TPA) and flavonoids (TFC) under the conditions tested.

In general, polar solvents (*e.g.*, water, ethanol, and acetonitrile) that easily react electrochemically in the spray nozzle are often used in electrospray ionization mass spectrometry (ESI-MS) experiments. These analyses were carried out in the negative ion mode because of its higher sensitivity in the detection of the distinct classes of phenolic compounds.

In total, 11 different phenolic compounds were identified. The data obtained for all peaks, including retention times, UV-*vis* spectra, pseudomolecular ions, and fragmentations obtained by MS^2 experiments, are presented in Table 4.7. Each compound was subsequently analyzed by MS. The identifications were confirmed by comparing the retention time and spectral data with those of reference compounds when available (*e.g.*, *p*-hydroxybenzoic acid, quercetin 3-*O*-glucoside, and caffeic acid).

In Fraction 1, the compounds F1-1 and F1-2, with λ_{max} at 276 nm and anion at *m/z* 483, revealed fragments corresponding to the loss of a galloyl (-152 u), a galloyl plus a molecule of water (-170 u), and another fragment of 212 u, this fragmentation pattern is characteristic of galloyl glucose derivatives (Boulekbache-Makhlouf *et al.*, 2013). Peak F1-3 ($[M-H]^-$ *m/z* 353) was positively identified as 5-*O*-caffeoylquinic acid in comparison with the commercial standard. Peak F1-4 ($[M-H]^-$ *m/z* 499) was tentatively identified as a gallotannin, based on its characteristic fragment ions, corresponding the base peak ion at *m/z* 169 to a gallic acid anion and the ion at *m/z* 313 to a galloyl glucose moiety (Boulekbache-Makhlouf *et al.*,

2010). Compound F1-5 was tentatively identified as eucaglobulin or globulusin B based on the pseudomolecular ion at *m/z* 497 and fragmentation ions at *m/z* 313 and 169 (Boulekbache-Makhlouf *et al.*, 2013).

Table 4.7: HPLC–DAD–ESI/MS data for phenolic compounds in leaves of *Eucalyptus globulus*.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification mg/g dry leaves
F1-1	4.12	276	483	331(20),313(18), 271(100),211(6), 169(7)	Digalloyl-glucose	1.38 ± 0.04
F1-2	5.03	276	483	331(19),313(17), 271(100),211(5), 169(5)	Digalloyl-glucose	3.97 ± 0.12
F1-3	6.44	322	353	191(100),179(23), 173(5),161(2)	5-*O*-Caffeoylquinic acid	1.01 ± 0.01
F1-4	14.16	282	499	439(12),313(25), 22(111),169(100)	Gallotannin	0.32 ± 0.01
F1-5	16.25	280	497	313(34),169(100)	Eucaglobulin/Globulusin B	1.05 ± 0.02
F2-1	17.51	353	477	301(100)	Quercetin-3-*O*-glucuronide	2.19 ± 0.02
F2-2	18.07	343	477	301(100)	Quercetin-*O*-deoxyhexoside	1.26 ± 0.01
F2-3	20.93	353	447	301(100)	Quercetin-3-*O*-glucoside	0.67 ± 0.01
F2-4	21.3	351	447	315(100)	Isorhamnetin-*O*-pentoside	0.27 ± 0.01
F2-5	22.55	363	447	315(100)	Isorhamnetin-*O*-pentoside	0.51 ± 0.01
F2-6	23.74	358	461	315(100)	Isorhamnetin-*O*-deoxyhexoside	0.24 ± 0.01

Identification and quantification obtained under the experimental run No. 8 (120 min, 25 °C and 0% of ethanol) of HAE due to better visualization of the peaks. Quantification of phenolic compounds are expressed in "mean ± standard deviation".

In Fraction 2, flavonols were the main compounds in all samples studied, especially those derived from quercetin (λ_{max} around 353 nm and MS^2 fragment at *m/z* 301) were particularly abundant. Two glycoside quercetin derivatives (F2-1 and F2-3) were positively identified according to their retention, mass, and UV-Vis characteristics compared to commercial standards, while F2-2, was tentatively identified as quercetin-*O*-deoxyhexoside. Another group of flavonols detected was isorhamnetin glycoside derivatives (F2-4, F2-5, and F2-6) according to their UV-Vis and mass spectra (all of them released an MS^2 product ion at *m/z* 315), differing by their λ_{max} and retention time (Barros *et al.*, 2012; Boulekbache-Makhlouf *et al.*, 2013).

Overall, five compounds (F1-1 to F1-5) were identified in fraction 1, recorded at 280 nm: two digalloyl-glucose derivatives, 5-*O*-caffeoylquinic acid, a gallotannin derivative, and a compound that can be eucaglobulin or globulusin B. In fraction 2, six compounds were identified (F2-1 to F2-6), recorded at 370 nm: quercetin-3-*O*-glucuronide, quercetin-*O*-deoxyhexoside, quercetin-3-*O*-glucoside, two isorhamnetin-*O*-pentoside, and isorhamnetin-*O*-deoxyhexoside.

The extracts showed a high concentration of gallotannins and flavonoids and a minor composition in phenolic acids. Regarding flavonoids, flavonols derivatives of quercetin and isorhamnetin were found in greater amounts.

In general, the phenolic profile of the extracts obtained is relatively similar to those reported in the literature (Gomes *et al.*, 2018; Teixeira *et al.*, 2019). Quantitative differences can be ascribed to the use of different techniques and solvents, regional varieties, and collection conditions. Some authors suggest that, for studies involving a more complete characterization of plant extracts, analyzes should be carried out using samples collected periodically in the same region, under the same conditions of collection and treatment, considering that there may be variability in the bioactive profile over time (Caleja *et al.*, 2017; Mahumane *et al.*, 2016).

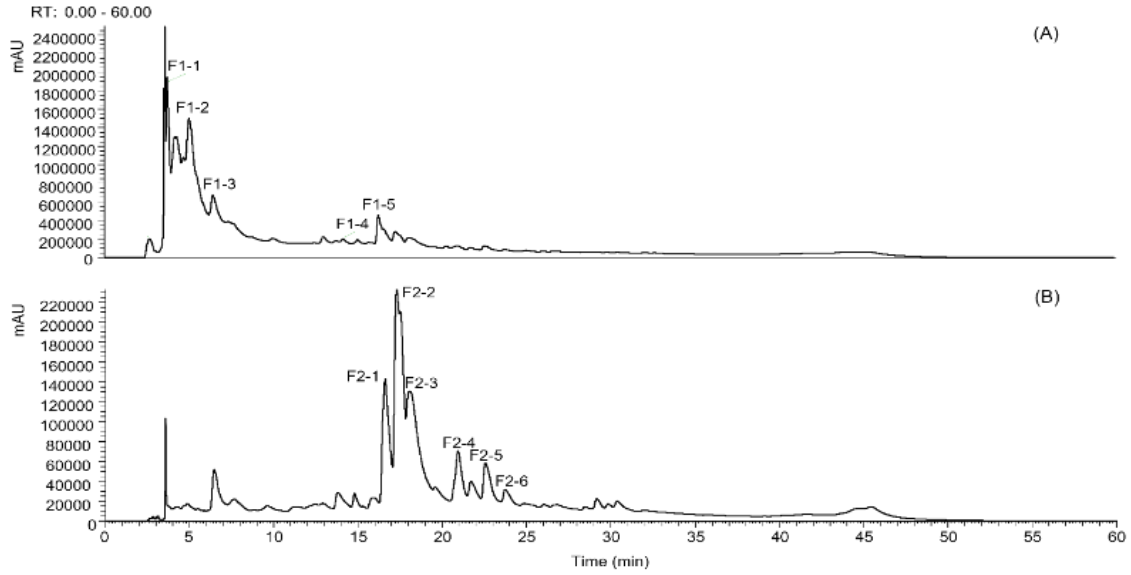


Figure 4.16: HPLC profile of phenolic compounds of the *E. globulus* extract (HAE) obtained under the experimental run No. 8 (120 min, 25 °C and 0% of ethanol) of HAE, recorded at 280 (A) and 370 nm (B). See Table 4.7 for peak identification.

4.4. Bioactive capacity

4.4.1. Antioxidant capacity

Extracts from plants and herbs have a wide variety of antioxidants compounds and mechanisms, thus more than one test is recommended to analyze antioxidant activity. Two assays were used to determine the influence of the extraction conditions of *E. globulus* leaves, using as a parameter the composition of the four optimal extracts evaluated (Y, TPA, TFC, and TPC). Lyophilized extracts were used in both tests.

TBARS assay quantified oxidative stress from peroxidative damage to lipids caused by free radicals. This lipid damage produces malondialdehyde (MDA), which reacts with 2-thiobarbituric acid (TBA) under conditions of high temperature and acidity, generating a chromogen that can be measured either by spectrophotometric or spectrofluorometric methods. The EC_{50}[5] concentration is visually located in the color change zone as indicated in Figure 4.17 and quantitative displayed in Table 4.8. Table 4.9 summarizes the analysis of variance performed to determine if there is any statistical difference between the mean values for the antioxidant capacity of the extracts. Based on this statistical inference, the null hypothesis (all extracts have the same effect) was rejected because the F value is greater than the F critical value and the P value is greater than the significance level ($\alpha = 0.05$). Control samples were Tris-HCl buffer solution and extraction solvent.

Table 4.8: Antioxidant capacities by TBARS for the optimal extracts.

Extract	TBARS (µg/mL)
Y	6.47 ± 0.08
TPA	5.67 ± 0.26
TFC	6.55 ± 0.18
TPC	5.59 ± 0.08

Values are expressed as "mean ± standard deviation". Y – Total Yield extract, TPA – Total Phenolic Acids extract, TFC – Total Flavonoids extract, TPC – Total Phenolic Content extract.

Table 4.9: Analysis of variance for the results of TBARS assay.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Response EC_{50}	6.750	3	2.250	76.866	3.5E-14	2.922
Error	0.878	30	0.030			
Total	7.628	33				

[5] Concentration that reduces by 50% the oxidants existing in the solution.

The results showed maximum antioxidant activity *in vitro* for the extract rich in phenolic compounds (TPC), which may suggest that this group of compounds plays an important role in the secondary metabolism of the plant and may be responsible for inhibiting lipid peroxidation in the cell lines tested. The results agree with those from *González-Burgos et al.* (2018), in which maximum protective effect against lipid peroxidation was observed for ethanol extract at 5 µg/mL concentration.

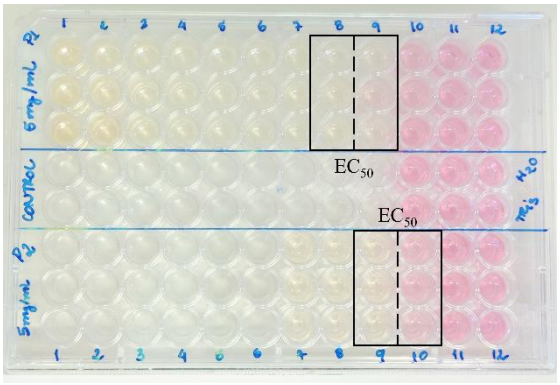


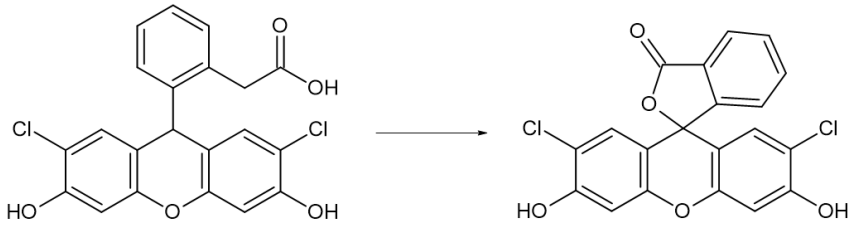
Figure 4.17: Lipid peroxidation assay (TBARS) in a 96-well plate with the samples in triplicate and visual positive result for MDA. The highlighted group of wells represents the zone of EC_{50}.

In a study by Gullón *et al.* (2019), the antioxidant capacity was directly affected by the concentration of ethanol in the solvent, where there was an increase in the antioxidant capacity with the increase in ethanol content up to approximately 50%. Solvent interference may explain why EC_{50} values for Y and TPA were higher (76.8 and 18.7% ethanol in the solvent), while for TFC and TPC they were lower, (33.5 and 25.0% ethanol in the solvent, respectively).

According to Kellett *et al.*, (2018), *in vitro* assays are used to analyze the antioxidant potential of extracts, but they may not accurately predict the antioxidant behavior in living systems. Therefore, it is of scientific importance to carry out cell-based assays, such as the Cell Antioxidant Activity assay (CAA).

CAA assay uses a fluorescent compound (DCFH) to monitor the inhibition of peroxyl radical-induced oxidation within the cell. 2,2'-Azobis(2-amidinopropane) dihydrochloride (ABAP) is added to the system as an inducer of free radicals, namely peroxyl radicals. Once the antioxidant enters the cell, it can prevent the DCFH from being oxidized to form

fluorescent DCF (Figure 4.18). Antioxidant capacity is therefore determined by intracellular fluorescence resulting from DCFH over time, the fluorescence being reduced compared to controls (Wolfe & Rui, 2007).



2′,7′-dichlorofluorescin
(DCFH - non fluorescent)

2′,7′-dichlorofluorescein
(DCF - fluorescent)

Figure 4.18: Oxidation of the 2′,7′-dichlorofluorescin (DCFH). Adapted from Kellett *et al.*, 2018.

The effectiveness of the cell lineage antioxidant treatments was quantified by examining the percent reduction in fluorescence. The values shown in Table 4.10 were generated by the fluorescence response readings for each treatment throughout the run and reveal a mild antioxidant capacity concerning the mechanism tested. The highest antioxidant capacity was found for extract Y, with a 40% reduction and corresponding to the extract with the highest mass yield, followed by TPA (15%). These data indicate that other classes of compounds can act directly on the plant's antioxidant potential and might work synergistically with phenolic compounds for this mechanism. The remaining samples did not return significant values at the concentration tested. Results of cellular antioxidant activity were not found in the literature for *E. globulus* leaves extracts tested with RAW 246.7 cell line.

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Table 4.10: Cellular antioxidant activity of hydroethanolic extracts of *E. globulus* leaves.

Extract	Maximum concentration tested (μM)	Inhibition for maximum concentration tested
Y	2000	40%
TPA	2000	15%
TFC	2000	>2000
TPC	2000	>2000

Y – Total Yield extract, TPA – Total Phenolic Acids extract, TFC – Total Flavonoids extract, TPC – Total Phenolic Content extract. % inhibition > 2000 μM – insufficient inhibition.

4.4.2. Antimicrobial activity

The antibacterial activity of eucalyptus leaf extracts was evaluated against three Gram-positive bacterial strains (*B. cereus*, *S. aureus*, and *L. monocytogenes*) and three Gram-negative (*E. Cloacae*, *E. coli*, *P. aeruginosa*, *S. enterica*, and *Y. enterocolitica*). For antifungal activity, the extracts were tested against *A. brasiliensis* and *A. fumigatus*. Different concentrations of the four extracts obtained under optimal conditions were tested to determine the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC) for each microorganism. Results are displayed in Table 4.11.

Table 4.11: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) (mg/mL) of extracts from *Eucalyptus globulus* leaves.

	E. globulus extracts								Positive control					
	Y		TPA		TFC		TPC		Streptomycin 1.0 mg/mL		Methicillin 1.0 mg/mL		Ampicillin 20 mg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
G(-) bacteria														
Enterobacter Cloacae	2.5	>10	2.5	>10	2.5	>10	2.5	>10	0.007	0.007	n.a.	n.a.	0.15	0.15
Escherichia coli	5	>10	5	>10	2.5	>10	2.5	>10	0.01	0.01	n.a.	n.a.	0.15	0.15
Pseudomonas aeruginosa	5	>10	2.5	>10	2.5	>10	2.5	>10	0.06	0.06	n.a.	n.a.	0.63	0.63
Salmonella enterica	5	>10	2.5	>10	5	>10	5	>10	0.007	0.007	n.a.	n.a.	0.15	0.15
Yersinia enterocolitica	2.5	>10	1.25	>10	1.25	>10	0.6	>10	0.007	0.007	n.a.	n.a.	0.15	0.15
G(+) bacteria														
Bacillus cereus	0.15	>10	1.25	>10	1.25	>10	0.6	>10	0.007	0.007	n.a.	n.a.	n.a.	n.a.
Listeria monocytogenes	1.25	>10	2.5	>10	0.6	>10	0.6	>10	0.007	0.007	n.a.	n.a.	0.15	0.15
Staphylococcus aureus	0.6	>10	0.3	>10	0.15	>10	0.3	>10	0.007	0.007	0.007	0.007	0.15	0.15

Y – Total Yield extract, TPA – Total Phenolic Acids extract, TFC – Total Flavonoids extract, TPC – Total Phenolic Content extract, G(+) – Gram-positive, G(-) – Gram-negative, n.a. – not applicable.
Maximum extract concentration tested of 10 mg/mL.

The results are in agreement with previously published data (Dezsi *et al.*, 2015; Fernández-Agulló *et al.*, 2015; Gullón *et al.*, 2017) and allow us to infer that eucalyptus leaf extracts can inhibit the growth of the tested strains. Among the Gram-negative bacteria, *E. coli*, *P. aeruginosa*, and *S. enterica* were strains that showed the highest resistance to the extracts. *S. aureus* was the gram-positive bacterium strain that returned the lowest MIC values and, therefore, high susceptibility to the extracts.

Considering the results for MIC and MBC tests, Gram-negative bacteria were more resistant than Gram-positive bacteria. This may be due to the layer of lipopolysaccharides present in the membrane of Gram-negative cells, which makes access to the membrane more restricted. In this sense, it is suggested that the antimicrobial activity of the extract is due to the interaction of phenolic compounds and flavonoids with the constituents of the cell membrane causing rupture and decrease in the fluidity of the bacterial membrane (Tsuchiya & Iinuma, 2000).

Antifungal activity was tested on filamentous fungi of the genus *Aspergillus*, *A. brasiliensis* and *A. fumigatus*, with positive results related to growth inhibition. Table 4.12 shows the results of minimum inhibitory concentrations (MIC) and minimum fungicide concentrations (MFC) for both strains, considering the extracts and ketoconazole as positive control. Maximum efficiency was reported for the Y extract for *A. fumigatus*, with a minimum inhibitory concentration of 1.25 mg/mL. There is scientific evidence that the antifungal effect of extracts is a result of the interaction of phenolic compounds with fungi membrane, resulting in their destabilization and eventual inhibition of the growth of *Aspergillus* species (Pizzolitto *et al.*, 2015). However, the extract that showed the most activity against the strains tested was the one corresponding to the total yield, indicating that other compounds present in this extract have a positive combined effect with the phenolic compounds already identified in this work (Table 4.7). Further studies should be conducted to confirm this hypothesis.

Table 4.12: Minimum inhibitory concentrations (MIC) and minimum fungicide concentrations (MFC) (mg/mL) of extracts from *Eucalyptus globulus* leaves.

	E. globulus extracts								Positive control	
	Y		TPA		TFC		TPC		Ketoconazole 1.0 mg/mL	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus brasiliensis	2.5	5	2.5	5	5	10	5	10	0.06	0.125
Aspergillus fumigatus	1.25	2.5	2.5	5	5	10	10	>10	0.5	1

Y – Total Yield extract, TPA – Total Phenolic Acids extract, TFC – Total Flavonoids extract, TPC – Total Phenolic Content extract. Maximum extract concentration tested of 10 mg/mL.

4.4.3. Cytotoxicity and anti-inflammatory activity

This colorimetric assay for the evaluation of cytotoxicity was based on Sulforhodamine B binding to the amino acids of cellular proteins (Figure 4.19), providing an estimate of the total protein mass and, by correspondence, the number of viable cells. Adherent cells were quantified by absorbance at a wavelength of 540 nm.

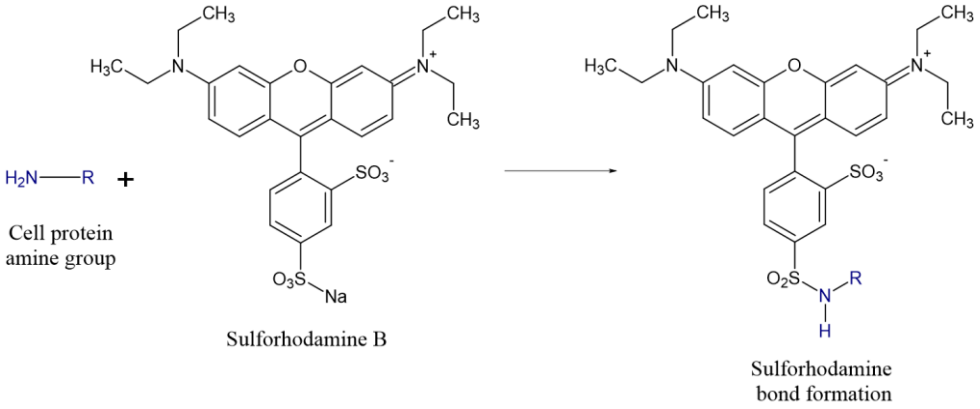

Figure 4.19: Sulforhodamine B reaction with amine-containing molecules producing stable sulfonamide bonds. Adapted from Hermanson, (2013).

The results are shown in Table 4.13, expressed in terms of GI_{50}, which corresponds to the minimum extract concentration to inhibit cell growth by 50%. The concentrations determined by this assay ranged from 8 to 69 µg/mL, in which the cell line showed high sensitivity to all evaluated extracts, which could be explained by the solid-liquid ratio used, returning a high concentrated extract compared to literature data (Teixeira *et al.*, 2019). Although the extracts demonstrate inhibitory capacity on the growth of the Vero cells, they might not demonstrate relevant toxicity for other lineages.

Recent studies presented significant cytotoxicity against tumor cell lines and cell lines infected with a virus, both treated with eucalyptus extracts. Teixeira *et al.* (2019) indicate that the extract of *E. globulus* leaves may limit the growth of NCI-H460 (lung carcinoma) cells by interfering with the cell cycle in growth and DNA synthesis phases, rather than by causing cellular apoptosis. Mota *et al.* (2012) state that the *E. globulus* extract seems to be efficient in reducing MDA-MB-231 (human breast cancer) cell growth capacity with an IC_{50} of 92.9 µg/mL. In Shang *et al.* (2016), *Eucalyptus robusta* (aerial parts) component, namely Eucalrobusone C, significantly blocked the proliferation in MCF-7 (breast adenocarcinoma)

cells and induced cell death through apoptosis, presented an IC_{50}[6] of 7.40 uM. In another study evaluating an isolated compound, namely Tereticornate C, Brezáni *et al.*, (2018) demonstrated the positive effect against the infectivity of HSV-1 virus in Vero cells and has no significant cytotoxic effects in uninfected cells.

In this work, the cytotoxic approach only determines the concentration that inhibits cell growth by 50% and does not consider the mechanism of interaction between the cells and the extract components, therefore, in-depth studies are recommended for a better understanding of the effect of these extracts on Vero and other cell lines.

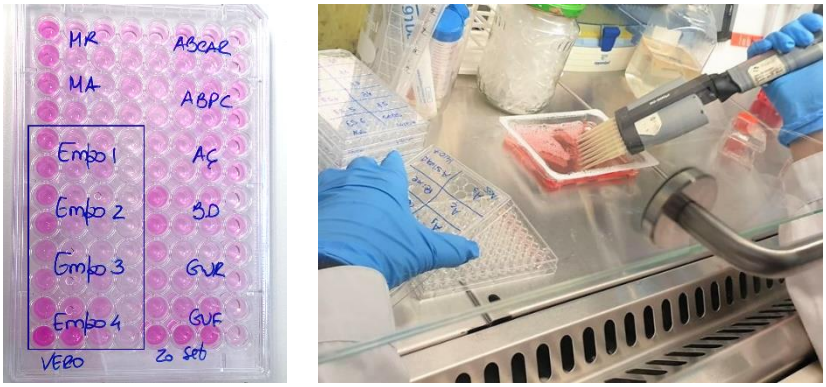

Figure 4.20: Cytotoxicity test in non-tumor Vero cell line performed on a 96-well plate and assay being performed on a laminar flow unit.

The anti-inflammatory capacity assay measured the inhibitory effects of *E. globulus* extracts against the inflammatory response caused by the nitric oxide (NO) level in RAW264.7 macrophages. Nitric oxide is a biological mediator that acts directly on the vascular and immune systems. When in quantities above normal, NO acts as a cytotoxic agent in pathological processes with inflammatory nature (Aktan, 2004).

As shown in Table 4.13, all extracts showed the ability to inhibit the production of nitric oxide at the concentrations tested, with IC_{50} values between 88-165 µg/mL being obtained (half maximum inhibitory concentration of NO production). Among the extracts, TPC showed the highest efficiency in inhibiting NO production. This could be related to its high concentration in phenolic compounds. The information available about the anti-inflammatory potential of eucalyptus extracts is quite scarce, as most authors report the

[6] Half maximal inhibitory concentration.

effectiveness of its essential oil. Some studies reported positive inhibitory activity of extracts in other cell lines and *in vivo* (Brezáni *et al.*, 2018; Sugimoto *et al.*, 2011; Vigo *et al.*, 2010).

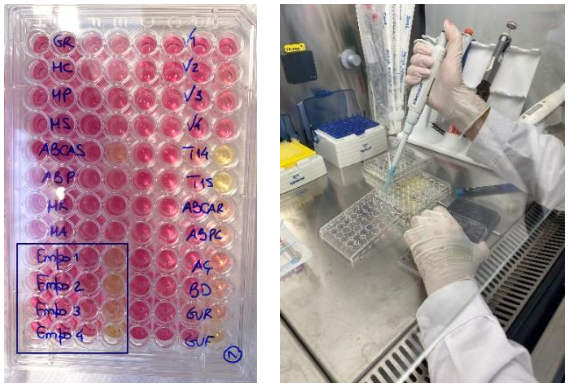

Figure 4.21: Anti-inflammatory test in RAW 264.7 cell line performed on a 96-well plate and assay being performed on a laminar flow unit.

Table 4.13: Cytotoxicity and Anti-inflammatory capacity of *E. globulus* leaves extracts.

	Extracts			
	Y	**TPA**	**TFC**	**TPC**
Cytotoxicity (GI_{50}, µg/mL)				
Vero	8.2 ± 0.2	69 ± 6	41 ± 4	22 ± 2
Anti-inflammatory activity (IC50, µg/mL)				
RAW 264.7	165 ± 8	156 ± 14	130 ± 3	88 ± 4

Y – Total Yield extract, TPA – Total Phenolic Acids extract, TFC – Total Flavonoids extract, TPC – Total Phenolic Content extract. Vero – African green monkey kidney, RAW 264.7 – mouse macrophage cell line. GI_{50} – concentration that inhibited 50% of cell growth. IC_{50} – half maximum inhibitory concentration of NO (Nitric Oxide) production. Ellipticin (positive control): 1.41 ± 0.06 µg/mL. Dexametasone (positive control): 6.3 ± 0.4 µg/mL.

5. Conclusions and future work

For a long time, natural products have been a promising source for the discovery of bioactive compounds, which have great importance for the development of new pharmaceutical, cosmetical, and food products and are widely used in traditional medicine. According to the presented literature review, this natural matrix is an important source of terpenoids and phenolic compounds, with analgesic, antifungal, antibacterial, anti-inflammatory, antiviral, and antioxidant activities, presenting low toxicity for the organism. Due to its high availability in the Portuguese territory, *E. globulus* is widely used in industry and the application of its extract has been increasing, both in Portugal and several other countries.

Methods, conditions, and the type of solvent are factors that can drastically alter the extraction yield, chemical profile, purity, and bioactivity of the extract. Despite the different methodology adopted by the authors, ultrasound and microwave-assisted extraction were the ones that showed the best efficiency overall. Heat-assisted extraction (maceration) and ultrasound-assisted extraction, using a mixture of ethanol:water as the solvent, were studied in this work, based on information available from previously published data.

In this work, ethanol content in the solvent and temperature were the most influential factors for optimal values using heat-assisted extraction. For ultrasonic-assisted extraction, the most influential factors were power and solvent, obtaining higher yields compared to the previous technique, using less ethanol in the solvent, and with shorter extraction time. The higher yield obtained by the UAE can be explained by the cavitation in the extractive medium. The presence of epicuticular wax present in the leaves obstructs the passage of these compounds and the cavitation phenomena accelerate mass transfer by the degradation of this wax layer. Therefore, for this plant matrix, preliminary wax removal procedures can be implemented to facilitate the obtainment of high added-value compounds. However, the incidence of ultrasounds in the extractive medium must be done with caution, as there is evidence that submitting *E. globulus* leaves to long periods of cavitation can lead to a reduction in the extraction performance due to the degradation of phenolic compounds, compromising the potential bioactivity of the extract.

For the extraction optimization, two RSM-based methods were used for modeling and analyzing the responses of interest influenced by three different variables (time, temperature

or power and solvent composition) to reduce the number of experiments and the cost of the operation. The dynamic RSM developed in this work can be considered an alternative approach to conventional methods, being effective in estimating optimal conditions. Further studies may be conducted to improve the robustness of this algorithm.

It was also verified that the phenolic profile for this variety of Eucalyptus is rich in phenolic acids and flavonoids, especially *p*-hydroxybenzoic acid and caffeic acid derivatives. Differences regarding the content of the compounds identified in this work and their correspondence in literature results can be attributed to the use of different techniques and solvents, regional varieties, and collection conditions. For studies involving a more complete characterization, studies should be performed on samples collected periodically in the same region, under the same conditions of collection and treatment, as there may be variability in the bioactive profile over time.

The leaves of *E. globulus* in this work returned high antioxidant, antimicrobial, anti-inflammatory potential, and low toxicity under the conditions analyzed. Most of the bioactive capacity of extracts seems to be strongly related to the phenolic profile. However, other non-phenolic compounds may be involved in the bioactive performance through different mechanisms.

It is important to mention that this work was done at a laboratory scale as a preliminary study of the bioactive characteristics of *E. globulus* leaves, considering the region, harvest time, and geoclimatic seasonality, hence that it may not represent the optimal parameters for industrial scale. For large-scale extractions, an in-depth study is necessary, considering all the variables that an industrial scope needs, namely operation time and solvent flow, for example. In extraction techniques at the industrial level, a large amount of solvent is used and considerably influences the environment in which it is discarded, production cost, and safety issues. The high toxicity and low biodegradability of volatile organic solvents led to the choice of a less harmful solvent such as ethanol:water, which has good extractive capacity and meets the emerging concepts of Green Chemistry, consistent with the search for eco-friendly solvents and processes.

Given the theoretical background and the literature review throughout this presented work, the application of green extraction techniques, as well as the detailed study of the methodology, extraction conditions, and mapping of the physical-chemical profile of the

extracts obtained are factors of fundamental importance for science and technology of natural products.

6. References

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